

Application No. 10/533,764  
Amendment Dated: December 4, 2006  
Reply to Office Action of October 2, 2006

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**Remarks/Arguments:**

**Status of the Application**

In the Office Action, claims 1-10, 13, 14, and 17-21 were allowed and claim 16 rejected. In the present Response, claim 7 has been amended so that claims 1-10, 13, 14, and 16-21 are pending.

Claim 7 has been amended to clearly identify the Formula I compound via the insertion of an "I" below the chemical structure depicted in claim 7 and not for reasons related to patentability. Support for this amendment can be found in the claims as originally filed and at page 7, lines 19-33. No new matter has been added.

**Rejections Under 35 U.S.C. § 112, 1st Paragraph**

Claim 16 has been rejected under 35 U.S.C. 112, first paragraph, as allegedly "failing to comply with the enablement requirement". The Office asserts that "[t]he claim(s) contain subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention", and as a result concludes "[s]uch a utility cannot be deemed enabled."

In alleging that the "scope of the claims includes treating anxiety for which there is no enabling disclosure", the Office points to page 12 of Applicant's specification which recites, "Compounds of the invention are useful for the treatment of diarrhoea, depression, anxiety, and stress related disorders such as post-traumatic stress disorders, panic disorders, generalized anxiety disorder, social phobia, and obsessive compulsive disorder, ...". To support the rejection, the Office relies on arguments that were set forth in the prior Non-final Office Action mailed April 17, 2006, wherein the Office addressed each of the following In re Wands factors: 1) the breadth of the claims; 2) the nature of the invention; 3) the state of the prior art; 4) the level of one of ordinary skill in the art; 5) the level of predictability in the art; 6) the amount of direction provided by the inventor; 7) the existence of working examples; and 8) the quantity of experimentation needed to make or use the invention based on the content of the disclosure. 858 F.2d 731 (Fed. Cir. 1988).

Applicants, however, respectfully assert that the Office has failed to establish a *prima facie* case of nonenablement because Applicants' application as-filed, when read in light of what was known in the art at the time such application was filed, enabled a person skilled in the pharmaceutical arts to use the full scope of the invention of Claim 16. Applicants further assert

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that such a result is inevitable when the evidence regarding each of the In re Wands factors is, in accordance with MPEP Section 2164.01(a), properly weighed. Indeed, as Section 2164.01(a) of the MPEP expressly indicates, "The determination that 'undue experimentation' would have been needed to make and use the claimed invention is not a single, simple factual determination"...but, "[r]ather, it is a conclusion reached by weighing all the above noted In re Wands factual considerations."

Applicants note that as Section 2164.08 of the MPEP indicates an "examiner should determine what each claim recites and what the subject matter is when the claim is considered as a whole, not when its parts are analyzed individually." Moreover, while "the specification must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation', ... not everything necessary to practice the invention need be disclosed ..." and "[i]n fact, what is well-known is best omitted." (See MPEP Section 2164.08) (citations omitted). Rather, "[a]ll that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art". (See MPEP Section 2164.08). In fact, "the scope of enablement must only bear a "reasonable correlation" to the scope of the claims." (See MPEP Section 2164.08) (citations omitted).

Applicants now turn to the arguments relied on by the Office in rejecting Claim 16 as allegedly nonenabled.

#### Breadth of the Claims

With regard to the breadth of the claims, the Office asserts that the "instant claim embraces millions of compounds with a benzhydryl scaffold with a variation of substituents at three different positions" and that such substituent variety gives "a diverse range of compounds, which provide different physical and chemical properties to the individual substituted benzhydryl scaffold." The Office also states that anxiety "can be defined as a complex combination as negative emotions that includes fear, apprehension and worry and is often accompanied by physical sensation such as palpitations, nausea, chest pain, and/or shortness of breath."

Applicants, however, direct the Office's attention to page 28, lines 4-14 of Applicants' specification, wherein Applicants expressly state the following:

The compounds of the invention are found to be active towards  $\delta$  receptors in warm-blooded animal, e.g., human. Particularly the compounds of the invention are found to be effective  $\delta$  receptor ligands. *In vitro* assays, *infra*, demonstrate these surprising activities, especially with regard to agonists potency and efficacy as demonstrated in the rat brain functional assay and/or the human  $\delta$  receptor functional assay (low). This feature may be related to *in vivo* activity and may not be linearly correlated with binding affinity. In these *in vitro* assays, a compound is

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tested for their activity toward  $\delta$  receptors and  $IC_{50}$  is obtained to determine the selective activity for a particular compound towards  $\delta$  receptors. In the current context,  $IC_{50}$  generally refers to the concentration of the compound at which 50% displacement of a standard radioactive  $\delta$  receptor ligand has been observed.

; to page 30, lines 7-19 of Applicants' specification, wherein Applicants expressly state the following:

The agonist activity of the compounds is measured by determining the degree to which the compounds receptor complex activates the binding of GTP to G-proteins to which the receptors are coupled. In the GTP binding assay, GTP[ $\gamma$ ] $^{35}S$  is combined with test compounds and membranes from HEK-293S cells expressing the cloned human opioid receptors or from homogenised rat and mouse brain. Agonists stimulate GTP[ $\gamma$ ] $^{35}S$  binding in these membranes. The  $EC_{50}$  and  $E_{max}$  values of compounds are determined from dose-response curves. Right shifts of the dose response curve by the delta antagonist naltrindole are performed to verify that agonist activity is mediated through delta receptors. For human  $\delta$  receptor functional assays,  $EC_{50}$  (low) is measured when the human  $\delta$  receptors used in the assay were expressed at lower levels in comparison with those used in determining  $EC_{50}$  (high). The  $E_{max}$  values were determined in relation to the standard  $\delta$  agonist SNC80, i.e., higher than 100% is a compound that have better efficacy than SNC80.

; and to page 31, lines 9-14 of Applicants' specification, wherein Applicants expressly state the following:

Based on the above testing protocols, we find that the compounds of the present invention and some of the intermediates used in the preparation thereof are active toward human  $\delta$  receptors. Generally, the  $IC_{50}$  towards human  $\delta$  receptor for most compounds of the present invention is in the range of 0.15 nM – 30.4 nM with an average of 2.30 nM. The  $EC_{50}$  and  $\%E_{max}$  towards human  $\delta$  receptor for these compounds are generally in the range of 2.4 nM -2325 nM and 60 – 114, respectively.

Applicants further direct the Office's attention to page 34, lines 7-8 wherein Applicants state, "For the anxiety and anxiety-like indications, efficacy has been established in the geller-seifter conflict test in the rat." Applicants point out that the "compounds of the invention" referred to in Applicants' specification at the very least included, as of the filing date, the 8 intermediate and 79 example compounds.

Applicants acknowledge that while not all of the exemplified compounds are encompassed by Formula I, a sufficient number of the exemplified compounds are encompassed by Formula I such that coupling the encompassed Formula I compounds with the *In vitro* testing procedures set forth at page 28, line 17 to page 31, line 24 and the *In vivo* geller-seifter testing procedure referred to at page 34, lines 7-8 provides a scope of enablement bearing a reasonable correlation to the scope of Claim 16. Accordingly, Applicants respectfully

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assert that the scope of enablement provided by the application to one skilled in the art as of the date such application was filed is commensurate in scope with the scope of protection sought by Claim 16.

#### Direction or Guidance

With regard to direction or guidance, the Office claimed "[t]hat provided is very little." The Office further claimed that "[t]here is no dosage range information in the Specification", and "[t]hus, there is no specific direction or guidance regarding a regimen or dosage effective specifically for the therapy of anxiety."

Applicants, however, indicated at page 13, lines 22-25 that "[t]he dosage will depend on the route of administration, the severity of the disease, age and weight of the patient and other factors normally considered by the attending physician, when determining the individual regimen and dosage level at [sic] the most appropriate for a particular patient"; at page 14, line 30 to page 15, line 2 that "[d]epending on the mode of administration, the pharmaceutical composition will preferably include from 0.05% to 99%w (per cent by weight), more preferably from 0.10 to 50%w, of the compound of the invention, all percentages by weight being based on total composition"; and at page 5, lines 3-5 that "[a] therapeutically effective amount for the practice of the present invention may be determined, by the use of known criteria including the age, weight and response of the individual patient, and interpreted within the context of the disease which is being treated or which is being prevented, by one of ordinary skills in the art." Additionally, as Applicants have already pointed out hereinabove, the application as-filed clearly stated that the 79 example and at least some of the 8 intermediate compounds disclosed therein were found to be active toward human  $\delta$  opioid receptors via the assays and testing protocols set forth therein, and even further that such compounds were found to be efficacious in the well accepted geller-seifter anxiety model. Accordingly, Applicants respectfully assert that the information disclosed in the application as-filed provided adequate guidance to the person of ordinary skill in the art as of the date the present application was filed.

#### Working Examples

With regard to working examples, the Office asserts that "[t]he Applicant has not provided any supporting documents, such as working examples or pharmacological testing, to indicate the said compounds are useful for the therapy of anxiety."

Applicants, however, are perplexed by this assertion because as Applicants have already pointed out hereinabove the application as-filed clearly stated that the 79 example and at least

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some of the 8 intermediate compounds disclosed therein were found to be active toward human  $\delta$  opioid receptors via the assays and testing protocols set forth therein. Moreover, the specification further states that "[f]or the anxiety and anxiety-like indications, efficacy has been established in the geller-seifter conflict test in the rat."

Applicants respectfully remind the Office that a "specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." MPEP Section 2164.04. Applicants respectfully assert that the Office has failed to provide any evidence that indicates there is reason to doubt the truth of the statements Applicants are relying on for enabling support. Accordingly, Applicants respectfully assert that in light of the exemplified compounds and statements indicating such compounds exhibited 1)  $\delta$  opioid receptor activity and 2) efficacy via the geller-seifter conflict test as to anxiety and anxiety-like conditions, Applicants provided working examples and pharmacological testing in the as-filed specification evidencing the compounds encompassed by Formula I are useful for the therapy of anxiety.

#### Skill of Those in the Art

With regard to skill of those in the art, the Office argued that "[t]he skills of those in the art haven't established that delta opioid receptor agonists can be used to treat anxiety." In reaching this conclusion, the Office relied on a 2004 article by Saitoh et al. as indicating "that as of 2004, the prospect that delta opioid receptor agonists would be anxiolytics is only a possibility, not an established fact."

Applicants, however, respectfully disagree and note that the aggregate of information known at the time the application was filed indicates that a person of ordinary skill in the art believed at the time the present application was filed that a correlation between the  $\delta$  opioid receptor and anxiety existed. Indeed, Applicants own pharmacological assays and protocols, which were disclosed in the present application as-filed and already discussed hereinabove, supported such a correlation as of the date the present application was filed. Moreover, as Applicants indicated in the present application as-filed, the accepted geller-seifter conflict test model established efficacy of the compounds as to anxiety and anxiety-like conditions. It is of import to note that the Geller-Siefter model was a well accepted model as of the date the present

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application was filed for identifying compounds efficacious in treating anxiety and anxiety related disorders.

With regard to the aggregate of information Applicants assert was known at the time the application was filed, Applicants direct the Office's attention to the various articles and blogs dated pre-November 7, 2002 being submitted herewith in Appendices A to I. More specifically, Applicants note that

- 1) the article Filliol et al., "Mice deficient for  $\delta$ - and  $\mu$ -opioid receptors exhibit opposing alternations of emotional responses", *Nature Genetics*, Vol. 25, pps 195-200 (June 2000) (attached hereto as Appendix A) indicates a correlation is believed to exist between delta opioid receptors and anxiolytic compounds;
- 2) the following three articles demonstrate that the endogenous ligand for delta receptors is anxiolytic:
  - i) Kang et al., "Overexpression of proenkephalin in the amygdala potentiates the anxiolytic effects of benzodiazepines", *Neuropsychopharmacology*, Vol. 22, issue 1, pps 77-88 (Jan. 2000) (attached hereto as Appendix B),
  - ii) Kang et al., "Changes in nociceptive and anxiolytic responses following herpes virus-mediated preproenkephalin overexpression in rat amygdala are naloxone-reversible and transient", *Annals of the New York Academy of Sciences*, Vol. 877, pps. 751-5 (June 29, 1999) (attached hereto as Appendix C), and
  - iii) Ragnauth et al., "Female preproenkephalin-knockout mice display altered emotional responses", Vol. 98, Issue 4, pps. 1958-63 (Feb. 13, 2001) (attached hereto as Appendix D);
- 3) the article Roberts et al., "Increased Ethanol Self-Administration in [delta]-Opioid Receptor Knockout Mice", *Alcoholism: Clinical and Experimental Research*, Vol. 25, Issue 9, pps 1249-56 (Sept. 2001) (attached hereto as Appendix E) indicates a correlation is believed to exist between delta opioid receptors and anxiety-like behavior;
- 4) the 1999 Science Blog from the American Chemical Society, "Novel molecule blocks pain receptor", <http://www.scienceblog.com/community/older/1999/A/199900178.html> (last visited 12/04/2006) (attached hereto as Appendix F) explains that studies in mice suggest the "opioid receptor-like 1" (ORL-1), which is a nerve receptor

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- widely distributed throughout the central nervous system, may play an important role in anxiety;
- 5) the 2001 Science Blog Roberts et al., "Probing the role of the delta opioid receptor in alcohol consumption", <http://www.scienceblog.com/community/older/2001/A/200110253.html> (last visited 12/04/2006) (attached hereto as Appendix G) indicated that genetically knocking out the delta opioid receptor in mice led to an increased state of anxiety in the mice;
  - 6) the on-line article found at [http://www.genome.ad.jp/dbget-bin/www\\_bget?omim+165195](http://www.genome.ad.jp/dbget-bin/www_bget?omim+165195) (last visited 12/04/2006) (attached hereto as Appendix H) explained that the anxiogenic- and depressive-like responses obtained in the OPRd1 -/- mice of Filliol et al. indicated that delta-receptor activity contributes to improvement of mood states (See Filliol et al article attached hereto as Appendix A); and
  - 7) the English language abstract of Dauge et al., "Study of induced effects by selective CCKB agonists cholecystokinin in the nociception and behavior in rodents", Therapie, Volume 47, Issue 6, pps. 531-9 (Nov. 1992) (attached hereto as Appendix I) suggests that delta opioid receptors mediate the anxiolytic effects of CCK agonists.

Applicants respectfully assert that when viewing the articles set forth in Appendices A to I in the aggregate, it is evident that persons of ordinary skill in the art were well aware of the correlation between anxiety and the delta opioid receptors at the time the present application was filed. Accordingly, Applicants respectfully assert that Applicants' specification, when viewed in light of what was known in the art (as evidenced by the articles attached hereto in Appendices A-I) at the time the present application was filed, enabled persons of ordinary skill in the art to use the compounds encompassed by Formula I to treat anxiety as of the filing date of the application.

#### Nature of the Invention and Predictability in the Art

With regard to the nature of the invention and predictability in the art, the Office asserted that "[t]he invention is directed toward medicine and is therefore physiological in nature". The Office further claimed that "[i]t is well established that 'the scope of enablement varies inversely with the degree of unpredictability of the factors involved,' and physiological activity is generally considered to be an unpredictable factor." (citations omitted).

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Applicants, however, respectfully assert that as the articles attached hereto in Appendices A to I indicate, a person of ordinary skill in the art was aware of the correlation between anxiety and delta opioid receptors as of the date the present application was filed. Moreover, as Applicants already pointed out hereinabove, the application as-filed clearly stated that the 79 example and at least some of the 8 intermediate compounds disclosed therein were found to be active toward human  $\delta$  opioid receptors via the assays and testing protocols set forth therein, and even further that such compounds were found to be efficacious in the well accepted geller-seifter anxiety model. As a result, Applicants respectfully assert that a person of ordinary skill in the art would be able to extrapolate the information disclosed in the specification and known in the art (as evidenced by the articles attached hereto as Appendices A-I) to the invention of Claim 16.

#### State of the Prior Art

With regard to state of the prior art, the Office asserted the "[t]hese compounds are substituted benzhydryl piperazines", and "[s]o far as the examiner is aware, no substituted benzhydryl piperazines of any kind have been used for the treatment of anxiety." In responding to Applicants previously submitted arguments, wherein Applicants relied on the article by Filliol et al. (attached hereto as Appendix A) as evidencing that a correlation between delta opioid receptors and anxiety was known at the time the present application was filed, the Office focused on the term "may" utilized by Filliol et al. and claimed such term "is suggestive of a possibility" and "doesn't imply enablement." The Office relied on Ex parte Bhide, which allegedly stated that "'one skilled in the art would understand the 'may be useful' and 'may also act as inhibitors' statements to be possibilities – not actual statements of use'" (citations omitted) to conclude that Filliol et al. "does not provide firm evidence that delta opioid receptor agonists are effective in treating a variety of anxiety."

Applicants, however, respectfully disagree and note that the aggregate of information known at the time the application was filed (as evidenced by the articles attached hereto as Appendices A to I) indicates that persons of ordinary skill in the art were well aware of the correlation between anxiety and the delta opioid receptors at the time the present application was filed. Moreover, Applicants respectfully assert that the Office is placing too much emphasis on Filliol et al.'s usage of the term "may" as scientific articles rarely use definitive terms in highlighting the lessons learned from the research detailed therein. On the contrary, it is well understood in the scientific community that definitive terms are not to be used in expressing any lessons learned in such scientific articles. Accordingly, Applicants respectfully assert that when



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the articles attached hereto as Appendices A to I are read in the aggregate it is abundantly evident that a person of ordinary skill in the art would have recognized at the time the present application was filed that a correlation between the opioid delta receptor and anxiety existed.

Quantity of Experimentation Needed

With regard to the quantity of experimentation needed, the Office argued that "[t]he quantity of experimentation needed would be an undue burden to one skilled in the pharmaceutical arts since there is inadequate guidance given to the skilled individual, regarding the pharmaceutical use, for the reasons stated above."

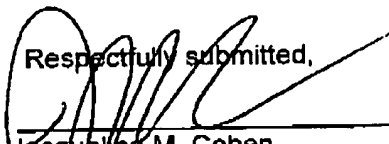
Applicants, however, again respectfully disagree with the Office and once again assert that the information disclosed in the specification as already discussed hereinabove would provide adequate guidance to a person of ordinary skill in the art so that any additional experimentation that may be needed to use a compound encompassed by Formula I to treat anxiety would not be undue.

In sum, Applicants respectfully assert that the Office has failed to establish a *prima facie* case of nonenablement because Applicants' application as-filed, when read in light of what was known in the art at the time such application was filed, enabled a person skilled in the pharmaceutical arts to use a compound encompassed by Formula I to treat anxiety. Moreover, Applicants respectfully assert that such a conclusion is inevitable when each of the In re Wands factors is properly evaluated and weighed. Accordingly, Applicants respectfully request the Office to withdraw this rejection.

**SUMMARY**

In view of the foregoing amendments and remarks, Applicants respectfully submit that in addition to previously allowed claims 1-10, 13, 14, and 17-21, Claim 16 is also in condition for allowance. In order to expedite disposition of this case, the Office is invited to contact Applicants' representative at the telephone number below to resolve any remaining issues. Although Applicants believe no fees are due, the Commissioner is hereby authorized to charge any deficiency in the fees or credit any overpayment to deposit account No. 26-0166, referencing Attorney Docket No. 100884-1 US.

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Patent App No: 10/533,764

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## APPENDIX A

## Mice deficient for $\delta$ - and $\mu$ -opioid receptors exhibit opposing alterations of emotional responses

Dominique Filliol<sup>1\*</sup>, Sandy Ghozland<sup>2\*</sup>, Johanna Chluba<sup>1\*</sup>, Miguel Martin<sup>2</sup>, Hans W.D. Matthes<sup>1</sup>, Frédéric Simonin<sup>1</sup>, Katia Befort<sup>1</sup>, Claire Gavériaux-Ruff<sup>1</sup>, Andrée Dierich<sup>3</sup>, Marianne LeMeur<sup>3</sup>, Olga Valverde<sup>2</sup>, Rafael Maldonado<sup>2</sup> & Brigitte L. Kieffer<sup>1</sup>

\*These authors contributed equally to this work.

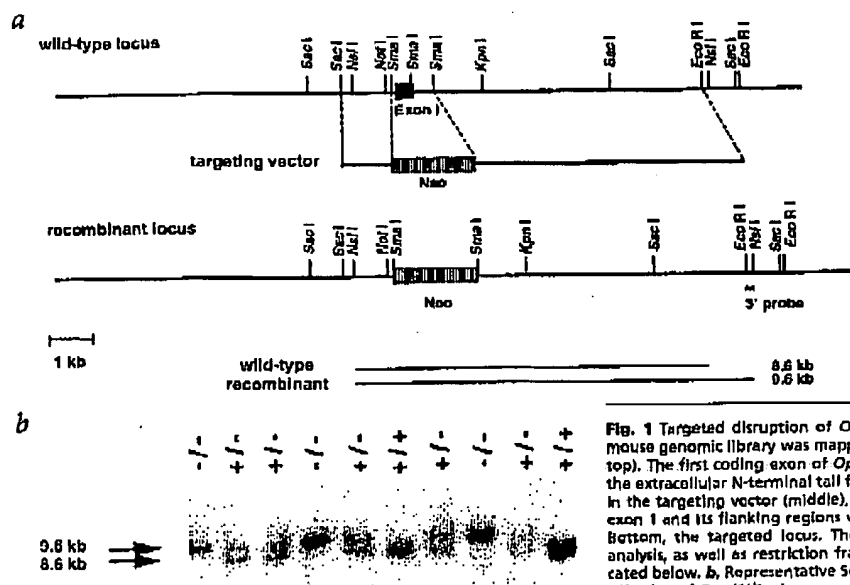
The role of the opioid system in controlling pain<sup>1</sup>, reward and addiction<sup>2,3</sup> is well established, but its role in regulating other emotional responses is poorly documented in pharmacology<sup>4</sup>. The  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors (encoded by *Oprm*, *Oprd1* and *Oprk1*, respectively) mediate the biological activity of opioids<sup>5</sup>. We have generated *Oprd1*-deficient mice and compared the behavioural responses of mice lacking *Oprd1*, *Oprm* (ref. 6) and *Oprk1* (ref. 7) in several models of anxiety and depression. Our data show no detectable phenotype in *Oprk1*<sup>−/−</sup> mutants, suggesting that  $\kappa$ -receptors do not have a role in this aspect of opioid function; opposing phenotypes in *Oprm*<sup>−/−</sup> and *Oprd1*<sup>−/−</sup> mutants which contrasts with the classical notion of similar activities of  $\mu$ - and  $\delta$ -receptors; and consistent anxiogenic- and depressive-like responses in *Oprd1*<sup>−/−</sup> mice, indicating that  $\delta$ -receptor activity contributes to improvement of mood states. We conclude that the *Oprd1*-encoded receptor, which has been proposed to be a promising target for the clinical management of pain<sup>8,9</sup>, should also be considered in the treatment of drug addiction and other mood-related disorders.

We inactivated *Oprd1* by deleting the first coding exon, including the translation-initiation codon (Fig. 1a). Animals homozygous

for the mutation were produced (Fig. 1b) at the expected mendelian frequency (wild type, 21%; *Oprd1*<sup>+/−</sup>, 53%; *Oprd1*<sup>−/−</sup>, 26%; *n*=261). *Oprd1*<sup>−/−</sup> mice were fertile, grew normally and showed no apparent developmental deficit.

Binding of  $\delta$ -selective radiolabelled compounds (Table 1) showed the absence of  $\delta$ -receptor binding sites from *Oprd1*<sup>−/−</sup> mice, confirming genetic ablation of  $\delta$ -receptors. In addition, the absence of [<sup>3</sup>H]DPDPE (81), [<sup>3</sup>H]deltorphin I (82) and [<sup>3</sup>H]naltrindole (81 and 82) binding of brain membranes from *Oprd1*<sup>−/−</sup> mice suggests that the described  $\delta$ -receptor pharmacological subtypes<sup>10</sup> arise from *Oprd1*. There was no obvious modification in the expression of  $\mu$ - and  $\kappa$ -receptor sites (ligand binding, Table 1), or in the proopiomelanocortin, preproenkephalin or preprodynorphin genes (*in situ* hybridization; data not shown), suggesting the lack of a major compensatory change within the opioid system. Similar observations were reported for another line of *Oprd1*-mutant mice<sup>11</sup>.

We examined spontaneous nociceptive thresholds in *Oprd1*<sup>−/−</sup> mice after application of a variety of acute noxious stimuli and found similar responses in wild-type and mutant mice (Table 2), in agreement with previous data<sup>11</sup>. This contrasts with *Oprm*<sup>−/−</sup> mice<sup>12</sup> and *Oprk1*<sup>−/−</sup> mice<sup>7</sup>, in which alteration of pain perception was detectable. Under non-stressful conditions, *Oprd1*<sup>−/−</sup> animals showed increased locomotor activity (Table 2), a behaviour which again differs from that of mice lacking *Oprm* (hypolocomotion<sup>6</sup>) or *Oprk1* (no change<sup>7</sup>). When exposed to stressful conditions of the open field, *Oprd1*<sup>−/−</sup> animals presented a shorter latency to first movement (which was significant on the first day of testing; data not shown), which was probably



**Fig. 1** Targeted disruption of *Oprd1*. **a**, A 14.5-kb fragment from a 129/SV mouse genomic library was mapped and partially sequenced (wild-type locus, top). The first coding exon of *Oprd1* is indicated by a black box and encodes the extracellular N-terminal tail followed by the first transmembrane domain. In the targeting vector (middle), a *Sma*I-*Sma*I fragment containing the entire exon 1 and its flanking regions was replaced by a Neo cassette (striped box). Bottom, the targeted locus. The 3' external probe used for Southern-blot analysis, as well as restriction fragments expected from a *Nsi*I digest, is indicated below. **b**, Representative Southern-blot analysis of mouse tail DNA from offspring of *Oprd1*<sup>+/−</sup> mice.

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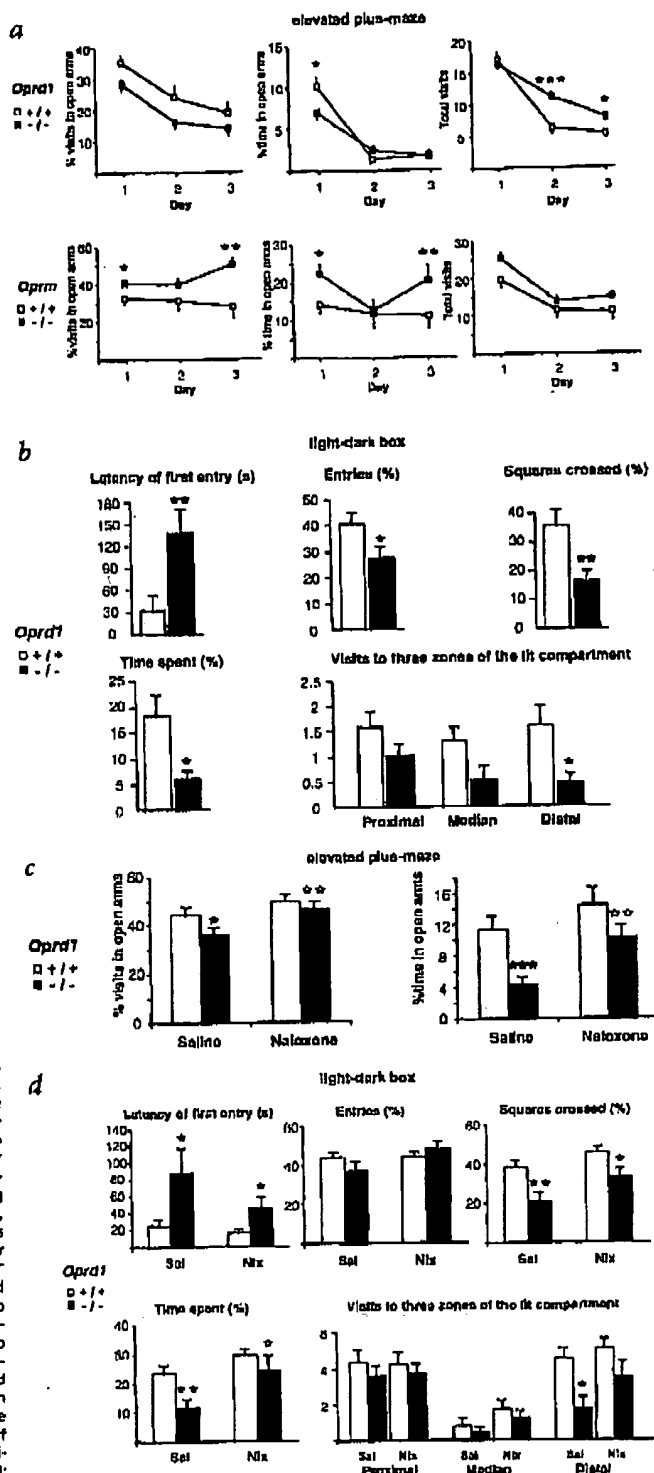
## letter

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partly due to their locomotor hyperactivity. No change was observed in any other response evaluated in the open field. In this test, neither *Oprm*<sup>-/-</sup> (data not shown) nor *Oprk1*<sup>-/-</sup> mice<sup>7</sup> behaved differently from their wild-type controls.

We then directly compared emotional responses of mice lacking *Oprd1*, *Oprm* or *Oprk1* in several paradigms. In a behavioural model of anxiety, the elevated plus-maze<sup>13</sup>, *Oprd1*<sup>-/-</sup> mice spent less time in the open arms and showed a constant trend toward a decreased percentage of visits to the open arms (Fig. 2a). We saw changes in both parameters in a second set of experiments (Fig. 2c, saline). In another model of anxiety, the light-dark box<sup>14</sup>, *Oprd1*-deficient mice showed an aversion to the lit compartment. We found differences between genotypes in all parameters in the first experiment (Fig. 2b), in accordance with the results obtained in the second set of experiments (Fig. 2d, saline). These modifications were independent from locomotor hyperactivity, which would tend to influence the parameters in an opposite way. Therefore, *Oprd1*-deficient mice showed higher anxiety in both the elevated plus-maze and the light-dark box, suggesting that the activity of *Oprd1*-encoded receptors may contribute to diminishing levels of anxiety. This is concordant with the increased anxiety reported in mice lacking preproenkephalin<sup>15</sup>. Together, these findings support the notion that tonic activation of  $\delta$ -receptors by endogenous preproenkephalin-derived peptides positively modulates anxiety states. The absence of marked behavioural alteration of *Oprd1*<sup>-/-</sup> mice in the open field may be due to inverse consequences of hyperlocomotor activity and higher levels of anxiety in this test.

In the elevated plus-maze, *Oprm*<sup>-/-</sup> mice behaved oppositely from *Oprd1*<sup>-/-</sup> mice (Fig. 2a), showing an increase in time spent and percentage of visits to the open arms. In the light-dark box, *Oprm*<sup>-/-</sup> mice also showed a tendency to respond differently from *Oprd1*<sup>-/-</sup> mice (shorter latency to first entry, longer time spent and



**Fig. 2** Anxiety-related behaviour. **a**, Elevated plus-maze. *Oprd1*<sup>-/-</sup> mice ( $n=25$ ) spent less time in open arms on day 1 compared with wild type ( $n=28$ ). The general activity was higher (total visits) on days 2 and 3, reflecting enhanced spontaneous locomotor activity. *Oprm*<sup>-/-</sup> mice ( $n=12$ ) visited more often and spent more time in the open arms compared with their wild-type littermates ( $n=14$ ). The total number of visits was not modified, presumably because hypolocomotion in these mice is too subtle to be detected under these conditions. *Oprk1*<sup>-/-</sup> mice showed no phenotype<sup>7</sup>. **b**, Light-dark box. Behaviour in the lit compartment is shown. The following parameters were evaluated: latency of first entry in seconds; entries, squares crossed and time spent in the lit compartment expressed as the percentage of total values (lit + dark compartment); and number of visits to the three different zones of the lit compartment. *Oprd1*<sup>-/-</sup> mice ( $n=14$ ) showed lower activity in the lit compartment compared with wild-type mice ( $n=14$ ) in all parameters evaluated. In the same test, *Oprm*<sup>-/-</sup> mice ( $n=14$ ) showed a trend towards the opposing phenotype (not shown) and *Oprk1*<sup>-/-</sup> mice ( $n=7$ ) behaved similarly to wild type (not shown). **c**, Effect of naloxone in the elevated plus-maze on day 1. As for untreated animals in (a), saline-treated *Oprd1*<sup>-/-</sup> mice showed decreased number of visits and time spent in the open arms (wild type,  $n=14$ ; *Oprd1*<sup>-/-</sup>,  $n=14$ ), and naloxone reversed this behaviour (wild type,  $n=13$ ; *Oprd1*<sup>-/-</sup>,  $n=14$ ). **d**, Effect of naloxone in the light-dark box. As in (b), saline-treated *Oprd1*<sup>-/-</sup> animals were less active in the lit compartment (wild type,  $n=14$ ; *Oprd1*<sup>-/-</sup>,  $n=13$ ), and naloxone (Nlx) tended to counteract this effect with an effect on percentage of time spent in the lit compartment (wild type,  $n=12$ ; *Oprd1*<sup>-/-</sup>,  $n=12$ ). Naloxone had no effect on wild-type mice in any of the tests. Values were analysed using a two-way ANOVA (except in c) followed by subsequent one-way ANOVA. Factors of variation were time (within subjects) and genotype (between subjects) in (a), and treatment (between subjects) and genotype (between subjects) in (b,d). Filled stars, differences between genotypes; open stars, effects of the treatment in the same genotype. One star,  $P<0.05$ ; two stars,  $P<0.01$ ; three stars,  $P<0.001$  (one-way ANOVA).

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Table 1 •  $\delta$ -,  $\mu$ - and  $\kappa$ -opioid receptor sites in *Opr1*-mutant mice

Radioligand <sup>a</sup>	Receptor selectivity	<i>Opr1</i> <sup>+/+</sup>		<i>Opr1</i> <sup>+/-</sup>		<i>Opr1</i> <sup>-/-</sup>	
		K <sub>d</sub> <sup>b</sup>	B <sub>max</sub> <sup>b</sup>	K <sub>d</sub>	B <sub>max</sub>	K <sub>d</sub>	B <sub>max</sub>
[ <sup>3</sup> H] Naltrexone	$\delta$	0.21±0.04	265±33	0.29±0.07	189±29	und	und
[ <sup>3</sup> H] DPDPE	$\delta 1$	1.32±0.12	131±5	1.98±0.56	82±5	und	und
[ <sup>3</sup> H] Deltorphin I	$\delta 2$	0.85±0.21	156±6	1.09±0.40	104±4	und	und
[ <sup>3</sup> H] DAMGO	$\mu$	0.51±0.09	154±30	0.71±0.06	187±17	0.73±0.10	170±8
[ <sup>3</sup> H] CI 977	$\kappa$	0.15±0.02	49±6	0.09±0.01	44±3	0.16±0.03	44±6

<sup>a</sup>Radioligands selective for each receptor type, as well as for each described  $\delta$  receptor pharmacological subtype, were used. <sup>b</sup>K<sub>d</sub> (nM) and B<sub>max</sub> (pmole/mg protein) were obtained by saturation analysis of radioligand binding on brain membranes and are mean±s.e.m. of 2–5 experiments performed in triplicate. Und, binding is undetectable. No  $\delta$ -receptor binding site was present in *Opr1*<sup>-/-</sup> mice and the total number (B<sub>max</sub>) of  $\mu$  and  $\kappa$  sites was not modified.

higher number of entries into the lit compartment; data not shown), although this was not significant in our statistical analysis. Therefore, in both tests, phenotypic changes in *Oprm*<sup>-/-</sup> mice were distinct from those observed in *Opr1*-deficient mice. The behavioural alterations in *Oprm*-deficient mice seemed subtler than those observed for *Opr1*-deficient mice and, in this respect, the few previous pharmacological studies had varying results. An anxiogenic-like activity of a  $\mu$ -agonist was reported<sup>16</sup>, in accordance with our findings, but an opposite response to  $\mu$ -agonists was also described<sup>17</sup>. Finally, *Oprk1*<sup>-/-</sup> mice showed no phenotype in the two anxiety-related behavioural tests (data not shown).

To investigate another aspect of mood-related behaviour, we examined responses of opioid-receptor-deficient mice in behavioural models of depression. In the forced swim test<sup>18</sup>, *Opr1*<sup>-/-</sup> animals showed a strong increase in the time of immobility (Fig. 3a), which was further confirmed in a second set of experiments (Fig. 3b, saline). This result suggests that lack of  $\delta$ -receptors may favour the establishment of a depressive-like behaviour. This is consistent with previous pharmacological studies showing antidepressant-like effects of endogenous enkephalins and  $\delta$ -agonists in the forced swim test, the conditioned suppression of motility and learned helplessness paradigms<sup>19,20</sup>. *Oprm*<sup>-/-</sup> mice showed slightly decreased immobility time (Fig. 3a), a response which differs from that of *Opr1*<sup>-/-</sup> mice. The forced swim test did not reveal any phenotype for *Oprk1*<sup>-/-</sup> animals (data not shown).

Depressive-like behaviour was also evaluated using the conditioned suppression of motility paradigm<sup>21</sup>. The conditioned response was attenuated in *Oprm*<sup>-/-</sup> mice, as shown by increased locomotion after foot-shock exposure compared with wild-type controls (Fig. 3c). We obtained a similar result in a second set of experiments. This suggests a decrease in the induc-

tion of depressive-like behaviour in *Oprm*<sup>-/-</sup> mice, an observation that opposes behavioural alteration of *Opr1*<sup>-/-</sup> mice in the forced swim test. *Opr1*<sup>-/-</sup> and *Oprk1*<sup>-/-</sup> mice exhibited no detectable phenotype in this test (data not shown). A putative depressive-like response in mutant mice would be difficult to detect under those conditions due to a ceiling effect in the conditioned response.

To investigate whether sex differences influence behavioural responses, we analysed data obtained for males and females separately in each animal model of anxiety and depression (Table 3). Our results show that the ensemble of phenotypic modifications is observed for males only, opening the possibility of sexual dimorphism in the activity of opioid receptors for these behaviours. The most significant alteration (increased immo-

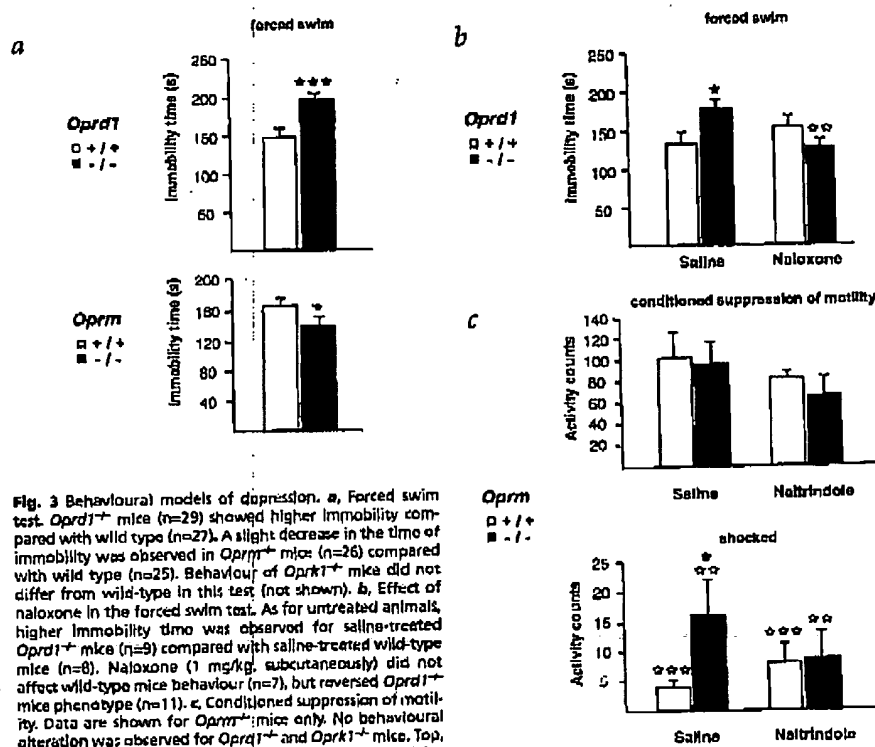


Fig. 3 Behavioural models of depression. **a**, Forced swim test. *Opr1*<sup>-/-</sup> mice (n=29) showed higher immobility compared with wild type (n=27). A slight decrease in the time of immobility was observed in *Oprm*<sup>-/-</sup> mice (n=26) compared with wild type (n=25). Behaviour of *Oprk1*<sup>-/-</sup> mice did not differ from wild-type in this test (not shown). **b**, Effect of naloxone in the forced swim test. As for untreated animals, higher immobility time was observed for saline-treated *Opr1*<sup>-/-</sup> mice (n=9) compared with saline-treated wild-type mice (n=8). Naloxone (1 mg/kg, subcutaneously) did not affect wild-type mice behaviour (n=7), but reversed *Opr1*<sup>-/-</sup> mice phenotype (n=11). **c**, Conditioned suppression of motility. Data are shown for *Oprm*<sup>-/-</sup> mice only. No behavioural alteration was observed for *Opr1*<sup>+/+</sup> and *Oprk1*<sup>-/-</sup> mice. Top, non-shocked animals showed similar locomotor activity, whether saline (wild type, n=8; *Opr1*<sup>-/-</sup>, n=9) or the  $\delta$ -antagonist naltrexone (2.5 mg/kg s.c.; wild type, n=10; *Opr1*<sup>-/-</sup>, n=7) was administered. Bottom, conditioning with electric foot-shocks induced severe immobilization in all animal groups. *Oprm*<sup>-/-</sup> mice (saline, n=10) were less affected than their wild-type controls (n=13). Naltrexone reversed this behaviour of mutant mice (n=10) without affecting wild-type mice (n=11). For all tests, filled stars indicate significant differences between genotypes, and open stars indicate significant effect of the treatment (forced swim test) or foot-shock exposure (conditioned suppression of motility) using two-way ANOVA between subjects followed by one-way ANOVA. One star,  $P < 0.05$ ; two stars,  $P < 0.01$ ; three stars,  $P < 0.001$ . *Opr1*<sup>-/-</sup> (9–11 per group) and *Oprk1*<sup>-/-</sup> (7–10 per group) did not behave differently from wild-type controls in this test (not shown).

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**Table 2 • Basal behavioural parameters in *Oprd1*<sup>+/+</sup> mice**

Nociceptive thresholds <sup>a</sup>		<i>Oprd1</i> <sup>+/+</sup>	n	<i>Oprd1</i> <sup>-/-</sup>	n		
tail immersion	tail withdrawal latency (s)	1.88±0.1	25	1.85 ± 0.1	33		
hot plate	licking time (s)	21.3±1.2	26	24.0±1.1	33		
	jumping latency (s)	167.1±12.8	26	140.0±10.7	33		
writhing	abdominal constrictions	16.7±3.6	8	18.3±3.3	11		
formalin	licking time (s)	65.7±6.4	9	67.1±6.3	10		
tail pressure	pressure (grams)	236.1±23.1	20	210.3±14.0	20		
Locomotion <sup>b</sup>		Day 1		Day 2		Day 3	
		+/+	-/-	+/+	-/-	+/+	-/-
<i>Oprd1</i>		887.1±62.8	1152.9±79.5 <sup>a</sup>	821.3±70.1	1170.8±73.7 <sup>d</sup>	670.5±54.6	1170.9±118.6 <sup>c</sup>
<i>Oprm</i>		1009.8±115.1	839.1±109.8	735.1±71.6	578.6±85.8	777.5±93.3	571.4±91.8 <sup>a</sup>
<i>Oprk1</i>		867.3±83.2	875.1±98.0	745.8±75.4	656.5±68.5	588.0±65.9	572.4±66.7

<sup>a</sup>No significant difference was detected between wild-type and mutant mice using one-way ANOVA analysis. n, number of animals. <sup>b</sup>Locomotor activity was measured in actimetry boxes. Statistically significant differences between genotypes (Dunnett test comparisons) were as follows: <sup>c</sup>P<0.001, <sup>d</sup>P<0.01, <sup>e</sup>P<0.05.

bility time in *Oprd1*<sup>-/-</sup> mice) was observed in both genders, however, indicating that modifications occur in females also.

Our data suggest that  $\mu$ - and  $\delta$ -receptors have opposite effects on mood states in these animal models, whereas  $\kappa$ -receptors do not seem to be involved. To further explore the respective contributions of  $\mu$ - and  $\delta$ -receptors, we examined the pharmacological action of opioids. The non-selective opioid antagonist naloxone modified the behaviour of *Oprd1*<sup>-/-</sup> mice in all tests, but had no effect on their wild-type controls. In the light-dark box, naloxone increased the activity of *Oprd1*<sup>-/-</sup> mice in the aversive compartment (Fig. 2d). In both the elevated plus-maze (Fig. 2c) and the forced swim test (Fig. 3b), naloxone-treated *Oprd1*<sup>-/-</sup> mice responded like wild-type mice, indicating that naloxone could reverse behavioural alterations of mutant mice in these tests. These data suggest that the endogenous activity of the remaining opioid receptors, presumably  $\mu$ -receptors, may be partly responsible for mood-related changes in *Oprd1*-deficient mice. Moreover, we found that the attenuation of conditioned suppression of motility in *Oprm*<sup>-/-</sup> mice was reversed by the  $\delta$ -antagonist naltrindole (Fig. 3c). In this case, the data indicate that a predominant activation of  $\delta$ -opioid receptors by endogenous peptides may be involved in the behavioural changes of *Oprm*<sup>-/-</sup> mice. Therefore, responses of mutant mice to the various opioid compounds suggest possible homeostatic interactions between  $\mu$ - and  $\delta$ -receptors in the regulation of mood states: a prevalent activity of  $\mu$ -receptors over  $\delta$ -receptors (*Oprd1*<sup>-/-</sup> mice) may facilitate anxiogenic and depressive-like behaviours, which may be otherwise inhibited when the activity of  $\delta$ -receptors is pre-

dominant (*Oprm*<sup>-/-</sup> animals).

Gene disruption in mice has recently proven to be a useful approach to explore the genetics of mood-related behaviours<sup>15,22</sup>. Our direct comparison of mice lacking each of the three opioid-receptor genes reveals that  $\mu$ - and  $\delta$ -opioid receptors act oppositely in regulating emotional reactivity. This highlights a novel aspect of  $\mu$ - and  $\delta$ -receptor interactions, which contrasts with the commonly accepted idea that activation of  $\mu$ - and  $\delta$ -receptors produces similar biological effects<sup>23,24</sup>. The neural circuits involved in this aspect of  $\mu$ - and  $\delta$ -receptor activity remain to be determined. The mesocorticolimbic system has a pivotal role in the control of emotional behaviour<sup>2</sup> and presents a high density of opioid receptors<sup>25</sup>. Behavioural alterations observed in *Oprd1*<sup>-/-</sup> and *Oprm*<sup>-/-</sup> mice most probably arise from the lack of specific receptors in this complex brain structure. Our data also show that the ablation of  $\delta$ -receptors is detrimental to mood states, an observation which may have therapeutic implications. Opiate addicts, who mainly abuse the  $\mu$ -opioid agonist heroin, present a high incidence of depressive disorders that seem to contribute to the maintenance of the addictive state. Also, the treatment of chronic pain states frequently includes antidepressant therapy<sup>26</sup>. Therefore, in addition to their potential analgesic activity,  $\delta$ -agonists may be useful in improving emotional states and, more generally, may be considered in the future as an alternative therapy to alleviate affective disorders.

**Table 3 • Gender analysis<sup>a</sup> of responses of mutant mice in behavioural models of anxiety and depression**

Behavioural test	Males		Females	
	<i>Oprm</i> <sup>+/+</sup>	<i>Oprm</i> <sup>-/-</sup>	<i>Oprm</i> <sup>+/+</sup>	<i>Oprm</i> <sup>-/-</sup>
Elevated plus-maze				
entries in open arms (%)	27.2±3.7	42.7±1.7 <sup>b</sup>	40.9±4.0	38.5±2.6
time in open arms (%)	12.5±3.4	24.3±3.3 <sup>c</sup>	16.2±2.3	19.8±3.4
total visits (no.)	20.1±3.3	27.7±2.2	19.0±1.2	22.2±2.0
Light-dark box				
latency of first entry (s)	17.1±3.5	168.3±38.0 <sup>b</sup>	61.0±48.3	62.0±24.0
entries (%)	41.7±4.7	22.4±5.5 <sup>b</sup>	38.4±5.8	37.1±7.4
squares crossed (%)	32.95±6.05	12.56±3.89 <sup>b</sup>	39.8±10.8	22.79±9.21
time spent (%)	14.76±3.06	5.08±1.80 <sup>b</sup>	23.0±10.0	7.68±3.63
Forced swim				
immobility time (s)	150.4±12.9	189.4±7.6 <sup>b</sup>	145±13.0	193.8±6.6 <sup>b</sup>
immobility time (s)	170.1±9.6	143.0±5.7 <sup>c</sup>	156.0±9.7	147.0±9.8
Conditioned suppression of motility				
non-shocked, activity counts	69.2±16.8	73.7±33.7	137.7±40.7	115.8±25.1
shocked, activity counts	2.72±1.16	24.0±9.3 <sup>c</sup>	6.20±1.59	12.85±5.1

<sup>a</sup>Results from experiments shown in Figs 2a,b and 3a,c were analysed considering males and females separately. The table summarizes parameters for which significant differences between wild-type and mutant mice appear in at least one gender. Results reported in the elevated plus maze were obtained on the first testing day. All behavioural alterations were observed for the male groups. In females, a modification was observed for *Oprd1*<sup>-/-</sup> mice in the forced swim test. Statistically significant differences between genotypes (one-way ANOVA) are indicated: <sup>b</sup>P<0.01, <sup>c</sup>P<0.05.

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## Methods

**Gene targeting.** We isolated a 14.5-kb *Oprd1* clone from a 129/SV mouse genomic library using a mouse *Oprd1* cDNA probe<sup>27</sup>. Mapping indicated that this clone contains the first coding exon, which encodes the amino-terminal region of the receptor and includes the ATG translation initiation codon. To construct the targeting vector, a 7.4-kb *NotI*-*EcoRI* fragment was excised and subcloned into pBluescript SK (Stratagene). A 0.9-kb *SmaI*-*SmaI* fragment containing the entire exon 1 was deleted and replaced by a 1.9-kb Neo cassette harbouring a Neo<sup>r</sup> gene under the control of a PGK promoter and a PGK polyadenylation signal. The insert was extended on the 5' end by insertion of a flanking *SacI*-*NotI* genomic fragment, resulting in a targeting vector with 1.2 kb and 6.4 kb homology to the wild-type locus at the 5' and 3' ends, respectively. The final construct was verified by DNA sequencing, linearized and electroporated into embryonic stem (ES) cells, as described<sup>28</sup>. Embryonic stem cells were derived at IGEMC from 129/SV mice. We screened 1,000 neomycin-resistant clones by Southern-blot analysis using a 3' external probe on *NsiI*-digested genomic DNA. We synthesized the probe from overlapping oligonucleotides derived from the *EcoRI*-*NsiI* region. A positive clone was identified and further probed using a 0.7-kb *SacI*-*SacI* fragment as 5' external probe, as well as a Neo probe, to control for proper integration of a single copy of the targeting construct (data not shown). The targeted ES cell line was injected into C57BL/6 blastocysts<sup>29</sup> and the resulting chimaeric males were mated with C57BL/6 females. We genotyped agouti pups by Southern-blot analysis, showing germline transmission. We generated *Oprm<sup>-/-</sup>* and *Oprk1<sup>-/-</sup>* mice by disruption of exon 2 in *Oprm* and deletion of exon 1 in *Oprk1*, respectively, as described<sup>6,7</sup>. All mice used here are homozygous mutant mice and their wild-type littermates under a hybrid 129 SV/C57BL/6 (50%/50%) genetic background.

**Ligand binding.** Membrane preparations from whole brain and binding were performed as described<sup>27</sup>. Total membrane proteins (100 µg per assay) were incubated in Tris-HCl (50 mM, pH 7.4) at 25 °C for 1 h with each radiolabelled ligand. We used [<sup>3</sup>H]-D-Ala<sup>2</sup>-MePhe<sup>4</sup>-Gly-oP<sup>5</sup> enkephalin (DAM-GO, Amersham, 54 Ci/mmol), enkephalin (2-D-Pen, 5-D-Pen), [tyrosyl<sup>2</sup>, 6-<sup>3</sup>H]-(N) (DPDPE, NEN, 45 Ci/mmol) and [<sup>3</sup>H]-D-Ala<sup>2</sup> deltorphin 1 (Zamco custom synthesis, 47 Ci/mmol 103600 dpm/picomole) at 0.05–5.6 nM concentrations, and [<sup>3</sup>H]-naltrindole and [<sup>3</sup>H]-[5R-(5α,7α,8α)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl)benzo[β]furan-4-acetamide (CI-977, Amersham, 47 Ci/mmol) at 0.0125–1.6 nM concentrations. Naloxone (Sigma) was used at a 2 µM concentration to determine non-specific binding. We performed experiments in triplicate using two (deltorphin 1) or three (others) distinct membrane preparations, each made from three brains for each genotype. We analysed binding data using the EBDA-LIGAND program (Biosoft).

**In situ hybridization.** Brains from wild-type, heterozygous and homozygous mutant mice were dissected and cryostat sections prepared (10 µm). We hybridized sections from two brains of each genotype with <sup>32</sup>S-labelled antisense riboprobes to detect mRNA encoding preproenkephalin, preprodynorphin and proopiomelanocortin. Probes and protocols have been described<sup>7</sup>. Similar staining patterns were obtained across brains and genotypes.

**Animal care.** We maintained animals under standard animal housing conditions in a 12-h dark-light cycle with free access to food and water. Animal care was in accordance with ethical guidelines (NIH, 1995; Council of Europe, 1996) and approved by a local ethical committee.

**Nociceptive thresholds.** For the tail immersion test, mice were maintained in a cylinder and their tail immersed in water at 52 °C; latency to tail withdrawal was measured. In the hot plate test, we placed mice on a hot surface heated at 50 °C and recorded the latency for paw licking and jumping. For the tail pressure, increasing pressure was applied to the tail of mice (tip diameter 1 mm) until a withdrawal response was elicited. In the writhing test, mice received 0.1 ml/10 g body weight of a 0.6% acetic acid solution by intraperitoneal route and the number of contractions of abdominal musculature (writhes) was counted from 5 to 15 min after the injection. In the formalin test, we injected 5% formalin (20 µl) subcutaneously into the dorsal surface of the right hindpaw and recorded the nociceptive response (time of paw licking) immediately over a 5-min period. These tests have been described<sup>7</sup>.

**Locomotor activity boxes.** We placed mice individually in actimetry boxes consisting of a plastic square area (25×25×25 cm, 100 Lux). The distance covered by the mouse was recorded by a videotracking system for a period of 10 min for 3 consecutive days.

**Open field.** Each animal was placed in an open-field apparatus<sup>7</sup> consisting of a rectangular area (70 cm wide×90 cm long×60 cm high) under bright illumination (500 Lux). We drew 63 squares (10 cm×10 cm) with black lines on the white floor of the apparatus. The parameters measured during the observation session of five min were the latency of crossing the first two squares from the central square where the mouse is initially placed; the number of squares crossed in the peripheral and central area of the field; entries into the central area; rearings; grooming bouts; defecation boli left in the field; and urination events. Mice were exposed to the test for three consecutive days.

**Elevated plus-maze.** The elevated plus-maze consisted of a black Plexiglas apparatus with four arms (16 cm long×5 cm wide) set in cross from a neutral central square (5 cm×5 cm). Two opposite arms were delimited by vertical walls (closed arms), whereas the two other opposite arms had unprotected edges (open arms). The maze was elevated 30 cm above the ground and placed in indirect light (100 Lux). At the beginning of the 5-min observation session, each mouse was placed in the central neutral zone, facing one of the open arms. The total numbers of visits to the closed arms and the open arms, and the cumulative time spent in the open arms and the closed arms were then observed on a monitor through a videocamera system (ViewPoint). An arm visit was recorded when the mouse moved all four paws into the arm.

**Light-dark box.** We used a box<sup>14</sup> consisting of a small (15 cm×20 cm×25 cm) compartment with black walls and a black floor dimly lit (5 Lux), connected by a 4-cm-long tunnel to a large compartment (30 cm×20 cm×25 cm) with white walls and white floor, intensely lit (500 Lux). We drew lines on the floor of both compartments to permit measurement of locomotor activity by counting the numbers of squares crossed. The lit compartment was separated by floor lines into three equal zones, from the tunnel to the opposite wall, named proximal, median and distal zones. Each animal was placed in the dark compartment facing the tunnel at the beginning of the observation session. Locomotor activity, time spent in and number of entries into each compartment, as well as the number of visits into each zone of the lit compartment, were recorded for 5 min.

**Forced swim test.** This test allows induction of a depressed state by forcing mice to swim in a narrow cylinder from which they cannot escape. We placed each mouse in a Plexiglas cylinder<sup>13</sup> containing water to a depth of 15 cm (21 °C–23 °C), so that the tail of the mouse could not reach the bottom of the apparatus. After a brief period of vigorous activity, the mice adopt a characteristic immobile posture that is reversed by the administration of compounds with antidepressant activity. Each animal was submitted to a forced swim during 6 min, and the total duration of immobility, including small maintenance movements, was measured during the last 4 min of the test.

**Conditioned suppression of motility.** In this test, we exposed mice to an electric foot-shock session in an apparatus consisting of a box with a grid floor (conditioning session). When re-exposed to the box, animals exhibit reduced locomotor activity and the time of conditioned immobility is classically sensitive to drugs with antidepressant activity<sup>21</sup>. Animals were submitted to electric foot-shocks (100 V, 200 ms, 0.1 Hz) in a 30 cm×30 cm×30 cm box by an isolated stimulator for 6 min, as reported<sup>22</sup>. The next day animals were re-exposed to the foot-shock box without receiving shocks and locomotor activity was measured for 6 min and expressed as the number of squares crossed and rearings during re-exposure to the box. To evaluate pain perception, we counted the number of jumps and vocalization bouts during the foot-shock exposure. No difference was observed in *Oprd1<sup>-/-</sup>* mice (number of jumps, wild type, 33.09±2.25; *Oprd1<sup>-/-</sup>*, 29.27±2.89; vocalization bouts, wild type, 23.55±1.81; *Oprd1<sup>-/-</sup>*, 20.82±2.31) or *Oprm<sup>-/-</sup>* mice (number of jumps, wild type, 25.08±1.78; *Oprm<sup>-/-</sup>*, 27.45±0.78; vocalization bouts, wild type, 21.50±1.91; *Oprm<sup>-/-</sup>*, 25.18±0.51), suggesting that pain perception was similar in all the groups under these experimental conditions. Additionally, the spontaneous nociceptive threshold was quantified in the tail electrical stimulation test as described<sup>30</sup>. No differences were observed between *Oprd1<sup>-/-</sup>* and *Oprm<sup>-/-</sup>* and their respective wild-type controls in the spontaneous responses using this nociceptive assay.

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**Drugs.** We administered naloxone and naltrindole (Sigma) by subcutaneous route in a volume of 10 ml/kg. All the compounds were dissolved in 0.9% saline and the drugs were injected 15 minutes before the test.

**Statistical analysis.** We made individual comparisons using a two-way ANOVA between subjects, followed by a one-way ANOVA. When a single factor was compared (genotype), a one-way ANOVA was used. The Dunnett test was used when post-hoc comparisons were required.

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## APPENDIX B

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# Overexpression of Proenkephalin in the Amygdala Potentiates the Anxiolytic Effects of Benzodiazepines

Wen Kang, Ph.D., Steven P. Wilson, Ph.D., and Marlene A. Wilson, Ph.D.

To elucidate the role of opioid peptides in control of the anxiety-like behavior and anxiety-reducing actions of benzodiazepines, a recombinant, replication-defective herpes virus (SHPE) carrying human preproenkephalin cDNA was delivered to rat amygdala. Viral infection resulted in a strong, localized transgene expression after 2–4 days which diminished after one week. Anxiety-like behavior and the anxiolytic effect of diazepam were assessed three days after gene delivery using the elevated plus maze test. While SHPE infection alone did not reduce anxiety-like behavior, rats infected with SHPE exhibited a greater response to the anxiolytic effect of diazepam when compared to rats infected

with a control virus (SHZ.1) containing the lacZ gene. The enhancement of diazepam action by SHPE was naloxone reversible, region-specific, and correlated with the time course of preproenkephalin expression. The findings implicate amygdalar opioid peptides in regulating the anxiolytic effects of benzodiazepines. This study also demonstrates the usefulness of recombinant herpes virus in evaluating the role of single gene products within specific brain sites in pharmacological responses and complex behaviors. [*Neuropsychopharmacology* 22:77–88, 2000] © 1999 American College of Neuropsychopharmacology. Published by Elsevier Science Inc.

**KEY WORDS:** Amygdala; Anxiety; Benzodiazepines; Opioid peptides; Gene transfer; Herpes virus vectors

In addition to its well known role in mediating fear and anxiety responses (Davis et al. 1994), the amygdala may also be a key neural substrate for the anxiolytic actions of benzodiazepines. Benzodiazepines (BZs) are believed to induce their anxiolytic properties by binding to GABA/benzodiazepine receptors in the brain (Haefely 1990). In addition, the amygdala has a high density of GABA/benzodiazepine receptors (Young and Kuhar 1980; Richards and Mohler 1984). Local infu-

sion of benzodiazepine agonists into the amygdala produces anxiolytic effects which can be blocked by co-administration of GABA<sub>A</sub> or benzodiazepine antagonists (Scheel-Kruger and Petersen 1982; Petersen et al. 1985; Pesold and Treit 1994, 1995). Furthermore, the anxiety-reducing actions of systemic benzodiazepines can be blocked by intra-amygdala injection of GABA/benzodiazepine antagonists (Sanders and Shekhar 1995).

The amygdala also has high levels of endogenous opioid peptides and opioid receptors (Loughlin et al. 1995), and opioid mechanisms have been implicated in many biological functions, including nociception, memory, and fear conditioning (Good and Westbrook 1995; McGaugh et al. 1996; Valverde et al. 1996). A partial anxiolytic action has been reported following microinjection of morphine into amygdala (File and Rodgers 1979).

Although opioid agonists are not used as anxiolytics in general, recent evidence strongly indicates a role for endogenous opioid peptides in the control of stress and anxiety (Olson et al. 1996). Preproenkephalin knockout

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mice have elevated level of anxiety-like behavior and aggression (Konig et al. 1996), and some novel  $\kappa$ -opioid agonists show anxiolytic properties (Privette and Terrian 1995). Several lines of evidence also indicate the involvement of endogenous opioid peptides in various aspects of benzodiazepine action, including their influences on food intake, locomotor activity, conflict, and anxiety-like behaviors (Millan and Duka 1981; Cooper 1983; Nowakowska and Chodera 1990). Interestingly, clinical studies suggest interactions between responses to benzodiazepines and opioids in humans (Darke et al. 1993). The anxiolytic effects of benzodiazepines can be blocked by opioid antagonists in both humans and laboratory animals (Billingsley and Kubena 1978; Koob et al. 1980; Duka et al. 1981, 1982; Agmo et al 1995; Tsuda et al. 1996).

Prior studies using herpes virus-mediated gene transfer demonstrated that overexpression of proenkephalin in amygdala produced antinociceptive effects, and illustrated the usefulness of this methodology for examining the role of neuropeptides in behavioral responses (Kang et al. 1998). To investigate if modifying opioid peptides in the amygdala altered anxiety-like behavior or the anxiolytic effects of benzodiazepines, a herpes virus vector (SHPE) expressing human preproenkephalin was delivered to the rat amygdala. Effects on anxiety-like behavior as well as the anxiolytic effect of diazepam were examined using the elevated plus maze test after the gene delivery.

## MATERIALS AND METHODS

### Recombinant Viruses

Initial stocks of replication-defective, ICP4-herpes viruses encoding either *lacZ* (SHZ.1; formerly named DZ) (Mester et al. 1995) or human preproenkephalin (SHPE) (Kang et al. 1998) under control of human cytomegalovirus immediate-early promoter/enhancer and the complementing 7B cell line were generously provided by J. C. Glorioso and M. A. Bender at the University of Pittsburgh.

### Subjects, Surgery, and Infection

This study was approved by the Institutional Animal Care and Use Committee of the University of South Carolina; the Principles for Care and Use of Laboratory Animals of NIH were followed in all experimental procedures. Adult male Long-Evans rats (Harlan) weighing 225–280 g were individually housed and maintained on a 12:12 h light/dark cycle with food and water available *ad libitum*.

Animals were handled daily and surgery was performed under phenobarbital anesthesia (65 mg/kg

Nembutal, i.p.). Rats were placed on the stereotaxic apparatus (David Kopf, Tjunga, CA), and the virus suspension was injected bilaterally into amygdala using a 10  $\mu$ l Hamilton syringe with a 26-gauge needle (Hamilton #80427) using the coordinates of AP  $-2.4$ , LM  $\pm 4.6$ , DV  $-8.5$  from bregma (Paxinos and Watson 1997). Viruses were suspended in cell culture medium with 10% glycerol. One microliter viral stock of SHPE or SHZ, containing about  $2 \times 10^6$  plaque forming units (pfu), was injected by hand at a speed of 0.2  $\mu$ l/min followed by 10 min diffusion period before needle withdrawal. Additional control groups received either bilateral virus injection in the caudate (coordinates: AP  $+0.5$ , LM  $\pm 3.5$ , DV  $-6.0$  from Bregma) (Paxinos and Watson 1997) or vehicle-injection (10% glycerol in cell culture medium) in the amygdala. Animals were allowed to recover after the surgery and monitored daily for general health (body weight, food and water intake, grooming behaviors).

For microinjection studies, bilateral 26-gauge guide cannulae precut to 9 mm below the pedestal (C315G; Plastics One, Roanoke VA) were aimed at the central amygdala using the same AP and LM coordinates as above, but lowered  $-7.7$  mm from the top of skull (Paxinos and Watson 1997). Guide cannulae were attached to the skull using skull screws and cranioplastic cement (Plastics One, Roanoke, VA), and guides were protected using dummy cannulae (C315DC; Plastics One, Roanoke, VA). Animals were habituated to removal of the dummy cannulae after surgery.

### Elevated Plus Maze Test

The elevated plus maze apparatus consisted of two opposing open arms (50  $\times$  10 cm) and two closed arms (50  $\times$  10  $\times$  40 cm high walls) connected with a center platform (10  $\times$  10 cm) and elevated 50 cm above the floor (Pellow et al. 1985). Each arm was marked into three equal divisions for better analysis of activity (entries into each of the three divisions). Tests were conducted either three or nine days after surgery. Thirty minutes before testing rats received an intraperitoneal injection of either vehicle (10% ethanol, 40% propylene glycol, i.p.) or diazepam (1 mg/kg; a generous gift of F. Hoffmann-LaRoche LTD, Basel Switzerland). This dose of diazepam produces sub-maximum increases in open arm measures in the plus maze (in our laboratory), thus allowing the observation of potentiation in these measures.

In some rats naloxone hydrochloride (5mg/kg, i.p.; Sigma Chemical Co., St. Louis, MO) was given simultaneously with diazepam. The elevated plus maze tests were conducted between 10:00 to 12:00 am during the early light part of the 12:12 light: dark cycle. At the beginning of each test session rats were placed in the center

platform with the head facing a closed arm, and allowed to freely explore for 10 min. The behavior was recorded by a videocamera mounted above the field, to allow the number of arm entries and time spent in each arm to be scored at a later time by an observer blind to the treatment condition. A reduced anxiety state was indicated by increased open arm activities, which were quantified as the amount of time that rat spent in the open arm relative to the total amount of time spent in any arm, as well as the number of entries into open arms relative to the total number of entries into any arm. The number of closed arm entries (all three divisions on both arms) was used as a measure of locomotor activity. Each animal was tested only once and those that showed less than 12 total entries (12 divisions on four arms) were excluded from data analysis. Rats were tested in several groups of eight to insure the reproducibility of the results.

To examine opioid-benzodiazepine interactions using alternate techniques, an additional study examined if local administration of the opioid antagonist naltrexone into the amygdala could attenuate the anxiolytic actions of diazepam. A higher, more effective dose (2 mg/kg) of diazepam was used in these experiments to allow assessment of naltrexone-induced attenuation in benzodiazepine effects. For microinjection studies, bilateral indwelling cannulae were positioned stereotactically (see above) 5–10 days before testing. Rats received bilateral injections of sterile saline (1  $\mu$ l) the day before testing to habituate them to the injection procedure. The day of testing, rats received bilateral injection of either sterile saline (1  $\mu$ l) or naltrexone (20  $\mu$ g in 1  $\mu$ l of sterile saline) using a 33-gauge internal cannula extending 1 mm beyond the guide cannula (C215I; Plastics One, Roanoke, VA). Injections were done over 1.5 min, and the internal cannula was left in place an additional 1.5 min while the animal was gently restrained. Immediately after microinjection, animals received 2.0 mg/kg diazepam (i.p.) and were tested for anxiolytic actions of diazepam on the elevated plus maze 30 min later as described above. Animals were perfused, and cannula placement was verified histologically. Only animals with both cannulae placed for injection into the central area of the amygdala were included in analysis.

#### Data Analysis

The behavioral measures were compared using analysis of variance (ANOVA) or *t*-test (experiments with only two groups). When overall ANOVAs reached statistical significance ( $\alpha = 0.05$ ), Bonferroni's multiple comparison tests were performed to assess specific group differences.

#### Histochemical Procedures

Immediately after testing, rats were sacrificed with an overdose of chloral hydrate and transcardially perfused

with phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.6). Brains were rapidly removed, post-fixed in the same solution overnight, and soaked in 15% sucrose in 0.1 M sodium phosphate (pH 7.6) for 24 hrs. Tissue sections were cut on a freezing microtome and processed for histological analysis. Brains infected with SHZ.1 virus (50  $\mu$ m sections) were reacted for  $\beta$ -galactosidase (Dobson et al. 1990) and counterstained with neutral red. Brains infected with SHPE virus (25  $\mu$ m sections) were mounted on silane-coated slides and processed for expression of human preproenkephalin mRNA by nonradioactive *in situ* hybridization using a modification of the procedure described in Paradies and Steward (1997). Only animals with confirmed needle placements were included in the data collection.

**Synthesis of Digoxigenin-labeled hPPE mRNA probes.** A plasmid designated pGEMhPPE was constructed by inserting human preproenkephalin cDNA obtained from plasmid pUR292- $\beta$ Gal-hPPE(1–267) (generously supplied by B.A. Spruce) (Spruce et al. 1988) into plasmid pGEM-1 (Promega, Madison, WI) between BamHI-HindIII sites. The plasmid was linearized with EcoRI or HindIII for transcription of sense and antisense mRNA, respectively.

The transcription mixture included 1  $\mu$ g of linearized template cDNA, 1 mM ATP, 1 mM GTP and 1 mM CTP, 0.65 mM UTP, 0.35 mM DIG-UTP, 10 mM DTT, RNase Inhibitor (2 U/ $\mu$ l of transcription mix), and SP6 or T7 polymerase (1 U/ $\mu$ l of transcription mix). Transcriptions were carried out at 37°C for 2 hrs. The DNA templates were then digested by adding 20 units of RNase-free DNase and incubated at 37°C for 15 min. The reaction volume was then adjusted to 100  $\mu$ l with H<sub>2</sub>O. The riboprobes were hydrolyzed at 60°C for 23 min by adding 100  $\mu$ l carbonate buffer (40 mM sodium bicarbonate/60 mM sodium carbonate). The probes were precipitated with 200  $\mu$ l of 200 mM sodium acetate/1% acetic acid, 100  $\mu$ g glycogen, plus 600  $\mu$ l cold 100% ethanol, and centrifuged at 4°C for 30 min. The pellets were washed once with 80% ethanol, dried, and suspended in H<sub>2</sub>O.

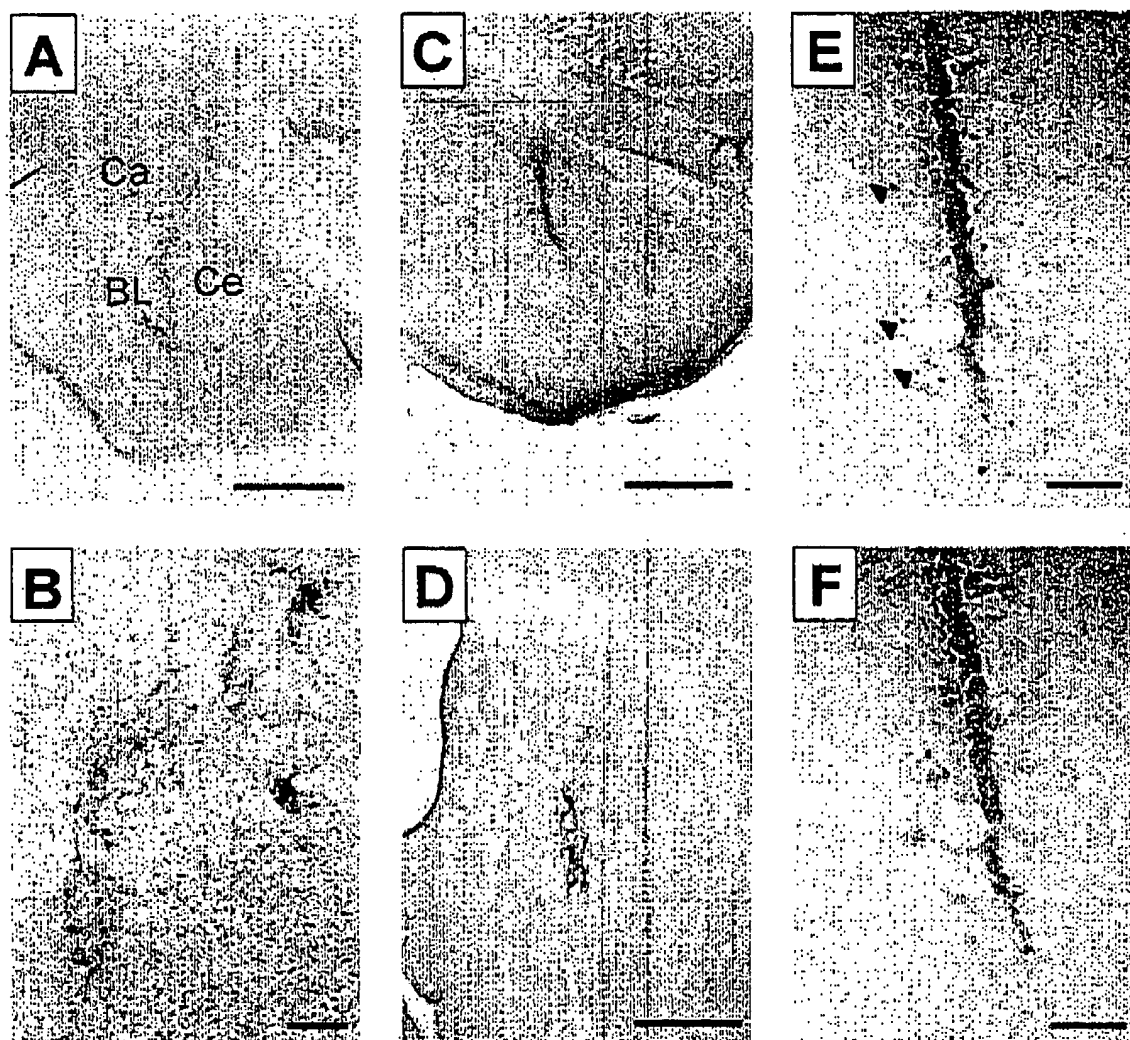
**In Situ Hybridization.** Twenty-five micron brain sections were mounted on silane-coated slides (Sigma Chemical Co., St. Louis, MO) and stored at –80°C. The sections were thawed at room temperature and fixed in 4% paraformaldehyde/0.1 M phosphate buffer for 10 min. After fixation the slides were rinsed twice with 0.5  $\times$  SSC (1  $\times$  SSC: 0.15 M NaCl/0.015 M NaCitrate, 10 min each), treated with proteinase K (in 0.5 M NaCl/10 mM Tris, pH 7.8) for 30 min, and rinsed twice with 0.5  $\times$  SSC (10 min each).

The sections were prehybridized for three hours at 42°C in hybridization buffer and RNA probes were then added to the sections (400–600 ng per section). The hy-

bridization buffer contained 50% formamide,  $2 \times$  SSC,  $1 \times$  Denhard's Solution, 10% Dextran Sulfate, 0.5 mg/ml yeast tRNA, 0.25 mg/ml salmon sperm DNA, and 0.5 mg/ml heparin. The hybridizations were carried at  $55^\circ\text{C}$  overnight in humid chambers which were equili-

brated with 50% formamide/ $4 \times$  SSC (Paradies and Steward 1997).

Stringency washes were performed as follows: twice in  $2 \times$  SSC/ $1 \text{ mM}$  EDTA at  $55^\circ\text{C}$  (10 min each), RNase ( $20 \mu\text{g/ml}$  in  $0.5 \text{ M}$  NaCl/ $10 \text{ mM}$  Tris, pH 8.0) at  $37^\circ\text{C}$



**Figure 1.** Herpes-mediated gene expression. (A) SHZ.1-induced expression of  $\beta$ -galactosidase in rat amygdala at three days after infection. (B) Higher magnification of amygdalar  $\beta$ -galactosidase expression seen in panel A. (C) SHZ.1-induced expression of  $\beta$ -galactosidase in rat amygdala at nine days after infection. Note the location of the needle tract, but the lack of  $\beta$ -galactosidase expression. (D) SHZ.1-induced expression of  $\beta$ -galactosidase in rat caudate at three days after infection. For Panels A-D, coronal sections ( $50 \mu\text{m}$ ) of paraformaldehyde-fixed brains were reacted with X-Gal and counterstained with neutral red. (E) and (F) SHPE-induced expression of hPPE mRNA. Coronal sections ( $25 \mu\text{m}$ ) of paraformaldehyde-fixed brains were taken two days after infection with SHPE and hybridized with digoxigenin-labeled human preproenkephalin specific mRNA probes. Adjacent sections from the same rat brain hybridized with human preproenkephalin mRNA anti-sense (E) or sense (F) probes, respectively. The arrowheads in Panel E indicate cells expressing the human preproenkephalin mRNA. Bars in Panels A, C, and D represent 1 mm, and bars in Panels B, E, and F represent 0.1 mm. Abbreviations: Ce, central amygdala; BL, basolateral amygdala; Ca, caudate.

for 30 min, twice in  $2 \times \text{SSC}/1 \text{ mM EDTA}$  at  $55^\circ\text{C}$  (10 min each), twice in  $0.1 \times \text{SSC}/1 \text{ mM EDTA}$  at  $65^\circ\text{C}$  (15 min each), and twice in  $0.5 \times \text{SSC}$  at  $55^\circ\text{C}$  (10 min each).

Digoxigenin immunohistochemical staining was performed as follows: slides were rinsed three times with TBS (400 mM NaCl/100 mM Tris, pH 7.5, 10 min each), blocked with 1% BSA/0.2% Triton X-100 in TBS for 1 hr, incubated with anti-digoxigenin Fab fragments (1:1000 dilution) conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN), rinsed twice with TBS (10 min each), and equilibrated once with NMT (0.1 M NaCl/50 mM  $\text{MgCl}_2$  in 0.1 M Tris, pH 9.5). The sections were then immersed overnight in colonization solution (3-Nitro Blue Tetrazolium salt 4.5  $\mu\text{l}/\text{ml}$  NMT, 5-bromo-4-chloro-3-indolyl phosphate 3.5  $\mu\text{l}/\text{ml}$  NMT; Boehringer Mannheim, Indianapolis, IN) at  $4^\circ\text{C}$ . The reaction was quenched by rinsing slides in 0.1 M Tris, pH 8.5.

## RESULTS

### Herpes-mediated Gene Expression

Histochemical analysis confirmed the virus-mediated transgene expression. As seen in Figures 1A and 1B, strong expression of  $\beta$ -galactosidase was seen in the first 2-4 days after SHZ.1 infection and declined thereafter, with little or no expression after one week (Figure 1C shows nine-day time point). The time course of this marker gene expression is similar to that observed in other studies (Glorioso et al. 1995).

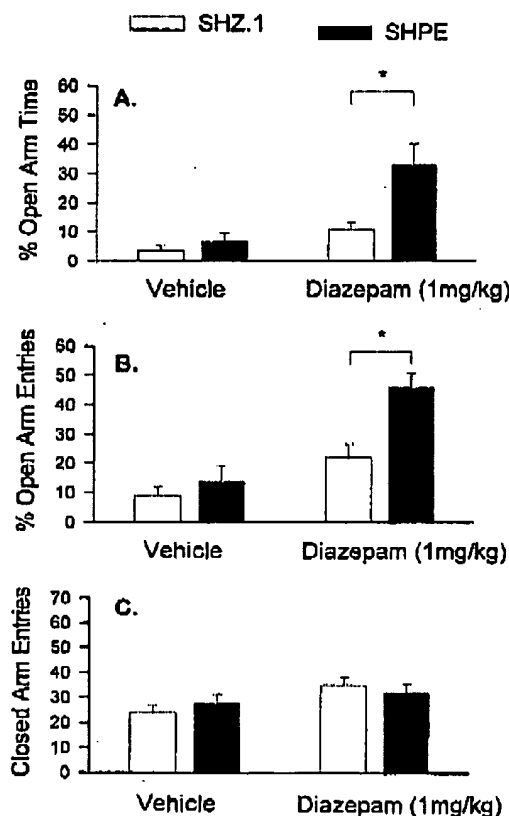
In rats infected with SHPE, human preproenkephalin mRNA expression peaked around day 2 (Figure 1E). While most of the infected cells were localized around injection sites, a few were also found along the needle tract. The target area for injection was the border between the central and basolateral nucleus, and anatomical analysis revealed that gene expression was predominantly in the central nucleus of the amygdala with some spread into basolateral region (see Figure 1A). The infected areas usually spread 1-1.5 mm around the injection site in the dorsal-ventral and anterior-posterior directions, but were much more limited in the medial-lateral direction.

Although expression of the SHZ.1 virus (Figures 1A and 1B) appeared more intense than SHPE mRNA expression (Figure 1E), visual analysis of the number of cell bodies infected revealed that similar numbers of neurons were infected using both procedures. The difference in apparent intensity is due to histochemical staining of  $\beta$ -galactosidase throughout the neuronal cell body and processes, as well as the use of thicker sections for this procedure compared with *in situ* hybridization (50 versus 25 microns). Virus-infected animals showed no overt behavioral or neurological abnormalities, and microscopic inspection of stained sections indicated neural damage from the viral infection was minimal. Although infected animals lost some body

weight immediately following stereotaxic surgery, food consumption, water intake, and normal grooming behavior suggested that the general health of the animals was not adversely affected by the viral infection.

### Potentiation of the Anxiolytic Effect of Diazepam by SHPE Infection in the Amygdala

A significant interaction between diazepam treatment and SHPE infection in the amygdala was seen in open arm exploration, as indicated by increases of percent



**Figure 2.** Potentiation of the anxiolytic effect of diazepam by SHPE-infection in the amygdala. Rats were infected with SHPE or SHZ.1 virus in the amygdala, and elevated plus maze tests were conducted three days after infection. Thirty minutes prior to testing, animals received either vehicle or diazepam (1 mg/kg, i.p.). The plus maze performance is presented as percent open arm time (A) percent open arm entries (B) and number of closed arm entries (C). Values represent mean  $\pm$  standard error of the mean (S.E.M.) of 10-11 rats in each group. \* significantly different from SHZ.1-infected animals. In Panel A, diazepam induced a significant increase of open arm time in SHPE-infected animals, and the response to diazepam was significantly higher in rats infected with SHPE compared to those infected with SHZ.1. Percent open arm entries showed similar results.

open arm time and open arm entries (Figures 2A and 2B), with SHPE infection in the amygdala significantly increasing the anxiolytic effects of diazepam treatment. As seen in Figure 2A, the percent open arm time was not significantly different between animals infected with SHPE and SHZ.1 that received a vehicle injection before testing. However, the increase of open arm time induced by diazepam injection was much greater in SHPE-infected animals, compared to SHZ.1-infected animals (Figure 2A) and animals receiving an intra-amygdala injection of vehicle (data not shown). Two-way ANOVA revealed an interaction between SHPE infection and diazepam treatment ( $F_{1,37} = 5.5$ ;  $p < .05$ ) for percent open arm time.

Following diazepam treatment rats infected with SHPE spent a significantly greater percentage of time in the open arm compared to those infected with SHZ.1 ( $t(18) = 3.7$ ,  $p < .01$ ) and SHPE-infected animals receiving a vehicle injection ( $t(18) = 4.4$ ,  $p < .001$ ). Percent open arm entries, an anxiety measure that factors out the activity level, showed similar results (Figure 2B), with ANOVA revealing a significant interaction between virus and diazepam treatments ( $F_{1,37} = 4.5$ ,  $p < .05$ ).

The locomotor activity, as indicated by total closed arm entries, was not affected by viral infection (Figure 2C;  $F_{1,37} = 0.01$  and  $F_{1,37} = 1.1$  for interaction,  $p > .1$ ). The enhancement in locomotor activity measures following low doses of diazepam is similar to previous results from our laboratory ( $F_{1,37} = 5$ ,  $p < .03$  for diazepam effect). Time in the center square did not differ between treatment groups, with values of  $22 \pm 5$ ,  $20 \pm 4$ ,  $23 \pm 6$ , and  $36 \pm 8$  mean  $\pm$  S.E.M. seconds spent in the center in the SHZ.1-Vehicle, SHZ.1-diazepam, SHPE-Vehicle, and SHPE-diazepam groups, respectively ( $F_{1,37} = 2$  for virus effect,  $F_{1,37} = 1$  for diazepam effect, and  $F_{1,37} = 1.5$  for interaction,  $p > .05$ ). Thus, animals spent more than 95% of the trial exploring the arms of the maze, although 8 of the 41 rats had zero values of percent open arm time or entries (during the 10 min trial). This low baseline level of open arm time was seen predominantly (7 of the 8) in two of the 10 batches of rats tested during these experiments, and were distributed throughout the SHZ.1 and SHPE-Vehicle groups (no zero open arm times were observed in the SHPE-diazepam treated groups). These results suggest expressing preproenkephalin in the amygdala greatly enhanced the anxiety-reducing effect of diazepam.

Although upon initial maze exposure five and ten minute trial times yield similar results (File et al. 1993), behavioral measures were also analyzed for the first 5 min of the 10-min test. Analysis of the first 5-min period of maze exposure showed a similar enhancement of the anxiolytic influences of diazepam in the elevated plus maze, as indicated by increased percent open arm time and percent open arm entries. Percent open arm times were  $6 \pm 2\%$  and  $13 \pm 6\%$  for SHZ.1 and SHPE vehicle

groups, and  $22 \pm 5\%$  vs.  $42 \pm 7\%$  for SHZ.1 and SHPE diazepam-treated rats, respectively. Percent open arm entries were  $11 \pm 4\%$  and  $17 \pm 7\%$  for SHZ.1 and SHPE vehicle groups, and  $25 \pm 5\%$  vs.  $49 \pm 4\%$  for SHZ.1 and SHPE diazepam-treated rats. Significant main effects of virus ( $F_{1,37} = 7$  for open arm time,  $F_{1,37} = 9$  for open arm entries,  $p < .02$ ) and diazepam ( $F_{1,37} = 20$  for open arm time and entries,  $p < .0001$ ) were observed, although the interaction terms were marginal ( $F_{1,37} = 3$ ,  $p = .09$ ). Post-hoc *t*-tests demonstrated that SHPE diazepam-treated rats differed from SHPE-vehicle groups for open arm time, while open arm entries of this group differed from both SHPE-vehicle groups and SHZ.1 diazepam-treated groups. This supports other work from our laboratory indicating that similar results are observed using both a five and ten minute trial period upon initial exposure to the maze.

#### Response to Diazepam Was Not Altered Following SHPE Infection in the Caudate

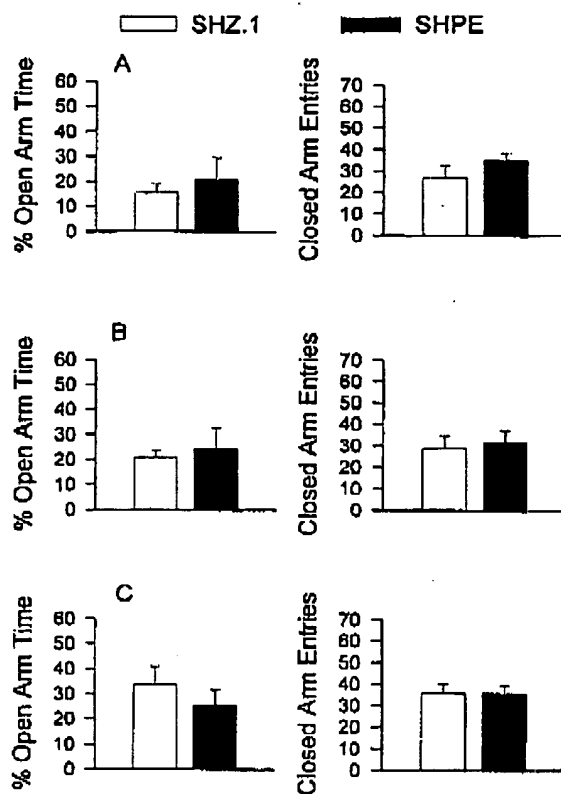
To examine the regional specificity of this potentiated diazepam effect, additional rats were infected bilaterally with either SHPE or SHZ.1 in the caudate nucleus (see Figure 1D). As shown in Figure 3A, the responses to diazepam were not affected by SHPE infection. Neither open arm time nor open arm entries (data not shown) differed between groups infected with SHPE and SHZ.1 in the caudate nucleus. These results indicate the potentiation of the anxiolytic effect of diazepam by SHPE was amygdala-specific.

#### The Enhanced Diazepam Action was Dependent on Preproenkephalin Gene Expression

Since transgene expression dissipated after one week, we examined the responses to diazepam in rats infected with either SHPE or SHZ.1 in the amygdala in the elevated plus maze test nine days after infection. The open arm time (Figure 3B) or open arm entries (data not shown) following diazepam injection no longer differed between rats infected with SHPE and those infected with SHZ.1. The closed arm entries remained unchanged. The results suggest the change in diazepam responses was due to SHPE-mediated expression of preproenkephalin, rather than nonspecific effects of viral infection.

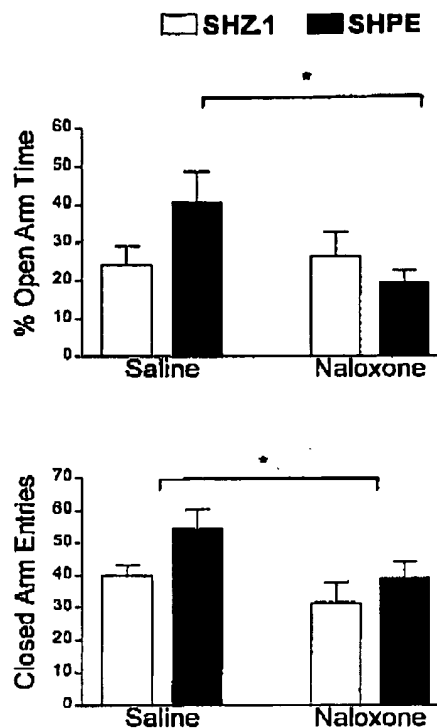
#### SHPE-Induced Enhancement of Diazepam Effect was Blocked by Naloxone

To insure an opioid-dependent mechanism was involved in this potentiated diazepam action, rats infected with either SHPE or SHZ.1 in the amygdala received intraperitoneal injections of the opioid antagonist naloxone hydrochloride (5 mg/kg) with diazepam (1 mg/kg) 30 min before testing. The opioid antagonist



**Figure 3.** Characterization of the response to diazepam following SHPE infection. All animals received diazepam injection (1 mg/kg, i.p.) 30 min before testing to examine the responses to diazepam. The elevated plus maze performance was presented as percent open arm time (left, Panels A, B, and C) and closed arm entries (right, Panels A, B, and C). Values represent mean  $\pm$  S.E.M. of 6–8 rats in each group, with direct comparisons between viral constructs in each experiment conducted with student *t*-tests (\*  $p < .05$ ). (A) Response to diazepam following viral infection in the caudate. Rats were infected with SHPE ( $n = 6$ ) or SHZ.1 ( $n = 8$ ) virus in the caudate nucleus and tested for their responses to diazepam in the elevated plus maze 3 days after infection. (B) Response to diazepam nine days after viral infection. Rats were infected with SHPE ( $n = 8$ ) or SHZ.1 ( $n = 8$ ) virus in the amygdala and tested for their responses to diazepam in the elevated plus maze nine days after the infection. (C) The enhancement of diazepam effect by SHPE infection in the amygdala was blocked by naloxone. Rats were infected with SHPE ( $n = 8$ ) or SHZ.1 ( $n = 6$ ) virus in the amygdala and tested three days later in the elevated plus maze. Naloxone (5 mg/kg, i.p.) and diazepam (1 mg/kg, i.p.) were injected 30 min before testing.

abolished the differences in diazepam responses between SHPE- and SHZ.1-infected groups (Figure 3C). Similar results were observed when data was analyzed during the first 5 min trial. This result indicates the po-



**Figure 4.** Effects of SHPE reversed by the opioid antagonist naloxone. Rats were infected with SHPE or SHZ.1 virus in the amygdala and tested three days later in the elevated plus maze. Naloxone (5 mg/kg, i.p.) or vehicle (saline, i.p.), plus diazepam (1 mg/kg, i.p.) were injected 30 min before testing in all animals. One animal in each of the four groups was tested concurrently. The elevated plus maze performance was presented as percent open arm time (top) and closed arm entries (bottom). For percent open arm time, ANOVA indicated a significant difference between groups, with post-hoc tests indicating SHPE rats receiving saline had higher percent open time than SHPE rats receiving naloxone. For closed arm entries (Panel B), naloxone decreased this measure of activity, while SHPE infected animals showed a slightly higher level of closed arm entries than SHZ.1 infected groups. There was no interaction, however, between viral infection and naloxone treatment. Values represent mean  $\pm$  S.E.M. of 8, 10, 9, and 8 rats in each group (from left to right), and \*  $p < .05$ .

tentiation of the anxiolytic effect of diazepam by SHPE was mediated by an opioid receptor mechanism.

Although this initial naloxone study indicated that no difference was observed between SHPE or SHZ.1-infected groups, the mean level of open-arm time in SHZ.1 diazepam-treated animals (see Figure 3C) was higher than that observed during our initial studies (Figure 1A). Since the influences of systemically administered naloxone on the anxiolytic actions of diazepam in



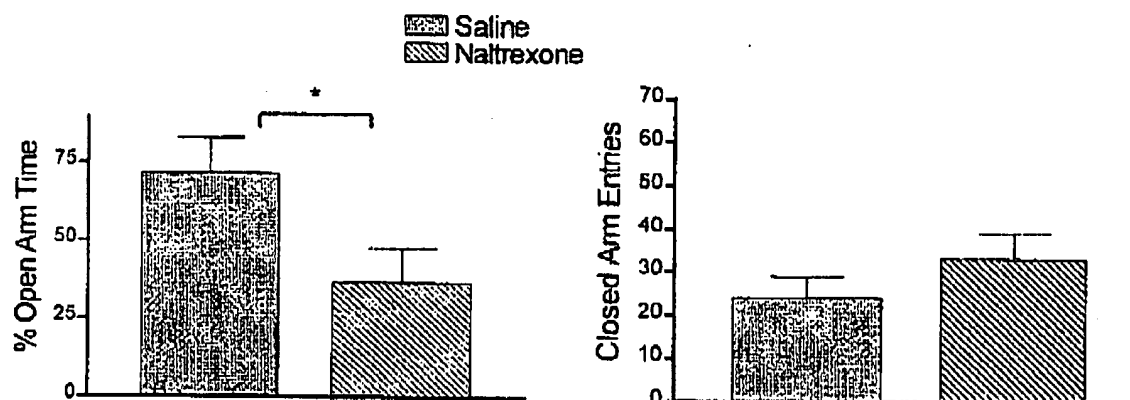


Figure 5. Effects of local naltrexone in amygdala on anxiolytic effects of diazepam. Non-infected rats received bilateral injections of naltrexone (20  $\mu$ g in 1  $\mu$ l of saline;  $n = 6$ ) or saline ( $n = 7$ ) into the amygdala through indwelling cannula just prior administration of diazepam (2 mg/kg, i.p.). Thirty minutes later animals were tested on the elevated plus maze. Open arm time (Left Panel) and closed arm entries (Right Panel) showed that this opioid antagonist administered locally in amygdala attenuated the anxiety-reducing influences of diazepam, as demonstrated by a reduction in percent open arm time. Closed-arm activity was not affected by naltrexone administration. Values represent mean  $\pm$  S.E.M. \* indicates significantly different ( $p < .05$ ) from saline animals.

the elevated plus maze appear variable (see Agmo et al. 1995, below), an additional naloxone reversal study was performed. Rats received either SHPE or SHZ.1 in the amygdala. Three days later rats received the opioid antagonist naloxone hydrochloride (5 mg/kg, i.p.) or saline, along with diazepam (1 mg/kg) 30 min before testing. The results in Figure 4 show that SHPE-infected group showed an increased anxiolytic action of diazepam (cf Figure 2) compared to SHPE-naloxone treated groups ( $F_{3,52} = 2.7$ ,  $p = .05$  for ANOVA on percent open arm time).

Naloxone administration had no effect on open arm time or entries in SHZ.1 infected groups. In both SHZ.1 and SHPE groups, naloxone administration decreased closed arm entries, suggesting a decrease in activity which was independent of viral construct (see Figure 4) ( $F_{1,31} = 4.6$ ;  $p < .04$ ). SHPE infection also enhanced closed arm entries slightly ( $F_{1,31} = 4$ ,  $p = .05$ ), but center square time was not affected by naloxone injection or viral infection (data not shown). These results support the notion that SHPE infection in amygdala potentiates the anxiolytic effects of diazepam, and that this effect is naloxone-reversible.

#### Naltrexone Administration in Amygdala Attenuates the Anxiolytic Actions of Diazepam

To further test the notion that amygdalar opioid peptides play some role in the anxiolytic actions of diazepam, the opioid antagonist naltrexone was microinjected into the amygdala of non-infected rats just prior to the systemic administration of an effective dose of diazepam (2.0 mg/kg, i.p.). As seen in Figure 5, local ad-

ministration of naltrexone [20  $\mu$ g; dose based on Kelley et al. (1996)] in the amygdala significantly attenuated the anxiolytic actions of diazepam ( $t(11) = 2.2$ ,  $p < .05$  for open arm time), but had no effect on activity ( $t(11) = 0.9$  for closed entries,  $p = .4$ ). These results from non-virally infected animals support the suggestion that opioid receptor-mediated events in the amygdala are important in the anxiety-reducing actions of diazepam.

## DISCUSSION

SHPE-mediated expression of preproenkephalin in the rat amygdala increased the behavioral responses to the anxiolytic effects of diazepam in the elevated plus maze test. This effect appeared to be region-specific because it was not found with SHPE infection in the caudate nucleus. The enhanced diazepam action was blocked by the opioid antagonist naloxone and disappeared after gene expression dissipated on day 9, indicating the behavioral changes were mediated by opioid peptides, and were not due to the influences of surgery or viral infection alone.

The findings indicate interactions between opioid peptides and the GABA/benzodiazepine system may play a major role in modulating the anxiolytic actions of benzodiazepines. This is also supported by the ability of the opioid antagonist naltrexone to attenuate the anxiolytic actions of diazepam in non-infected rats. Furthermore, the results demonstrate the importance of the amygdala as a neuroanatomical substrate for the anxiolytic effects of benzodiazepines, since altering a single

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protein in this brain structure caused such a remarkable change in the pharmacological response to diazepam. We have previously shown that transfer of the preproenkephalin gene into amygdala is antinociceptive in the formalin test (Kang et al. 1998). Taken together, these studies demonstrate the use of the herpes-mediated gene transfer technique to evaluate the functions of gene products within specific brain sites in regulating pharmacological responses and complex behaviors.

Although several lines of evidence suggest a strong association between endogenous opioid peptides and the GABA/benzodiazepine system in the brain, the specific mechanisms underlying the ability of preproenkephalin expression in amygdala to enhance the anxiolytic effects of diazepam are unclear. Potentiated diazepam actions could be related to upregulation of GABA/BZ receptors, enhanced allosteric GABA/BZ receptor coupling, and/or increased GABA release induced by enkephalin overexpression in amygdala. GABA and opioid neuropeptides are colocalized in many brain regions, including the central nucleus of amygdala (Roberts 1992; Veinante et al. 1997), and diazepam can modulate opioid peptide release in distinct brain regions (Duka et al. 1979; Wuster et al. 1980). Thus, if the anxiolytic actions of benzodiazepines relies on the release of endogenous opioids, this effect might be potentiated by virus-mediated overexpression of preproenkephalin. Additionally, chronic opioid treatment causes an alteration of GABA/benzodiazepine receptor numbers in the brain (Lopez et al. 1990; Rocha et al. 1993) suggesting potential changes in the GABA/BZ receptor could account for enhanced diazepam effects. Critically testing the involvement of such changes following gene transfer may be difficult due to the potentially small magnitude of effects limited to a confined brain area. Further, there is the possibility that such changes may not be limited to the central regions of amygdala, but may involve enhanced enkephalin release in amygdalar projections.

Studies examining the anatomical localization and neurotransmitter makeup of the neuronal population infected with the virus will also be critical to understanding the mechanism of preproenkephalin-induced increases in diazepam action. This is an important consideration since specific nuclei in the amygdala and in other nearby brain regions appear to play differing roles in various models of anxiety and effects of anxiolytics (Pesold and Treit 1995; Gonzalez et al. 1996; Davis et al. 1997; Moller et al. 1997).

Many neurons in the central amygdala express neuropeptides including enkephalin (see Petrovich and Swanson 1997; Horkaniemi et al. 1992), and it is currently unclear if neurons expressing human preproenkephalin are normally enkephalinergic, or if opioid peptides have been added to their neurotransmitter repertoire. Moreover, neurons of the lateral region of

the central nucleus have strong projections to the medial portion of the central nucleus, as well as the parabrachial nucleus and the bed nucleus of the stria terminalis (Petrovich and Swanson 1997). This lateral region is generally part of the amygdalar region affected by the infection, suggesting that enkephalin overexpression could be modifying these intra-amygdalar circuits. The results, at the very least, suggest that the infected amygdalar neurons are part of a neural circuit that can influence the anxiolytic actions of benzodiazepines. One advantage of this gene transfer methodology, compared with microinjection studies, is that the altered neuropeptide must be packaged, processed and released during a behavioral task, otherwise altered gene expression would not affect pharmacological responses. In contrast, microinjection of opioids into this region might activate receptors and modify circuits which can alter benzodiazepine actions, but are not critically involved in responses to these drugs.

Systemically administered naloxone abolished the enhanced effects of induced by SHPE, although naloxone alone did not modify diazepam responses in SHZ.1-infected animals. While others have found that opioid antagonists have no intrinsic effects on anxiety-like behaviors, a few reports show an anxiogenic action of opioid antagonists in the social interaction test and conflict paradigm (Agmo et al. 1995; Tsuda et al. 1996; Zhang et al. 1996). Opioid antagonists have been shown to block the anxiolytic or anti-conflict effects of benzodiazepines in humans and laboratory animals (Billingsley and Kubena 1978; Koob et al. 1980; Duka et al. 1981, 1982; Agmo et al. 1995; Tsuda et al. 1996), although some reports fail to show influences of opioid antagonists on the anticonflict properties of benzodiazepines (Britton et al. 1981). The underlying mechanisms and site of action for these effects remain unknown. In contrast, systemically-administered naloxone potentiates the anxiolytic action of sub-effective doses of benzodiazepines and buspirone (Belzung and Agmo 1997), suggesting that the influences of opioids may depend on the dose or type of benzodiazepine administered or the test of anxiety-like behavior. The apparently contradictory influences of opioid agonists (preproenkephalin overexpression) and antagonists (systemic naloxone) (Belzung and Agmo 1997) in this task, while both indicating opioid involvement, may also be related to the local versus systemic modulation of opioid actions. This is supported by the ability of locally administered naltrexone in the amygdala to attenuate the anxiolytic actions of systemically administered diazepam. Further studies will be needed to assess if this dose of naltrexone may have affected opioid processes by spread into additional areas and/or induced anxiogenic-like effects on its own. These studies, however, suggest this method of locally manipulating endogenous opioid release in confined brain regions may help elucidate the

role of opioids in select brain regions in modifying anxiety-like behaviors and responses to anxiolytics using other models of anxiety.

Transfer of the preproenkephalin cDNA into the amygdala selectively increased the response to the anxiolytic effect of diazepam. Closed arm entries, which are an indicator of locomotor activity, remained unaffected by various treatments among all the groups or were similarly affected by viral infection with SHZ.1 or SHPE (cf. Figure 4). Similar center times were also observed. Systemic diazepam administration produces anxiolytic actions in the elevated plus maze test as well as many other behavioral tests, and doses ranging from 2 to 5 mg/kg are often used (also see saline group in Figure 5). In our study, 1 mg/kg diazepam (given 30 min before testing) did not produce a significant effect in SHZ.1-infected animals or sham-injected animals, supporting the notion that this is a sub-effective dose in this behavioral test (Agmo et al. 1995; Griebel et al. 1996). Although analysis of the first five minute trial produced a greater apparent effect of diazepam on measures of anxiolytic behavior in SHZ.1 infected animals, both the five and ten minute analysis of plus maze data demonstrated that SHPE infection in the amygdala enhances the anxiety-related influences of diazepam in the plus maze task.

These studies demonstrate that both the transgene expression and the behavioral changes induced by herpes virus-mediated gene transfer were transient, peaking at 2-4 days after infection and dissipating by 7-9 days after injection in the amygdala. There were no apparent behavioral abnormalities following infection, and microscopic analysis did not demonstrate signs of neuronal damage. Similar responses to diazepam were observed in both SHZ.1-infected and vehicle-injected animals, suggesting these effects are not related to consequences of viral infection alone. The short post-operative recovery time may have contributed to the lower open arm activity in rats tested on day 3 compared to those tested on day 9, although other factors (batches of animals or viral preparations) may have also contributed to the alterations in baseline responding in the SHZ.1-infected groups tested at these two time points. Although modifying the viral construct to achieve long-term expression would be beneficial for future studies, the transient nature of transgene expression will also allow re-testing of infected animals after viral expression has waned (as in Carlezon et al. 1997).

In summary, the current findings demonstrate that expressing opioid peptides in the amygdala potentiated the anxiolytic effects of benzodiazepines. The fact that naloxone was able to block this potentiation suggests the effect is mediated by SHPE-induced increase of opioid peptides, the release of which is most likely triggered by the injection of diazepam. The ability of herpes virus-mediated gene transfer to alter preproenkephalin in en-

dogenous neural circuits could help elucidate opioid-benzodiazepine interactions and the role of opioid systems in various benzodiazepine actions. The effects of virus-mediated expression of preproenkephalin on other benzodiazepine effects, and on the anxiety-reducing actions in other animal models, may elucidate the mechanism underlying this opioid-mediated enhancement of benzodiazepine anxiolytic action.

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## APPENDIX C

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## Changes in Nociceptive and Anxiolytic Responses following Herpes Virus-mediated Preproenkephalin Overexpression in Rat Amygdala Are Naloxone-reversible and Transient

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To investigate the role of amygdalar opioid peptides in the control of anxiety, the anxiolytic effects of benzodiazepines, and nociception, a herpes virus vector (SHPE) expressing human preproenkephalin was stereotactically delivered to the rat amygdala. Studies examined viral expression and behavioral responses at 3–4 days or 9–10 days postinjection, and the ability of systemic injections of the opioid antagonist naloxone to block the actions of enkephalin overexpression in rat amygdala.

### VIRAL EXPRESSION

The recombinant SHPE virus was constructed by inserting the human preproenkephalin cDNA under control of the human cytomegalovirus promoter into the thymidine kinase locus. Control animals received a similar replication-defective SHZ.1 virus that contains lacZ.<sup>1</sup> Adult male rats (225–280 g) received SHZ.1 virus, SHPE virus ( $2 \times 10^6$  pfu, 1  $\mu$ L), or vehicle (10% glycerol in culture medium) injected bilaterally into the amygdala (AP-2.4, LM  $\pm$ 4.6, DV-8.5 from bregma) under phenobarbital anesthesia. After behavioral analysis, rats were sacrificed for histological analysis of needle placement,  $\beta$ -galactosidase expression in SHZ.1-infected rats, and human preproenkephalin mRNA expression by nonradioactive *in situ* hybridization in SHPE-treated rats (ref. 1 and Kang *et al.*, submitted).

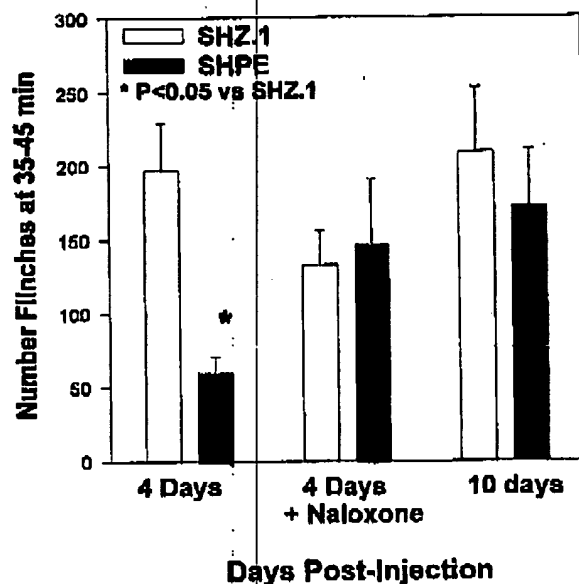
Compared with sham-injected animals, SHPE- and SHZ.1-infected animals showed no apparent behavioral or neurological abnormalities except for slight weight loss, and microscopic inspection suggested neural damage from the viral infection was minimal.<sup>1</sup> Viral infection in the amygdala resulted in strong, localized expression of the gene products in the first 2–3 days with little or no expression after one week (Kang *et al.*, submitted). Histochemical analysis confirmed that gene expression was predominantly in the central nucleus of the amygdala with some spread into the basolateral region (ref. 1 and Kang *et al.*, submitted). This was confirmed

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**FIGURE 1.** Four days after SHPE injection into the amygdala, rats show a reduced number of flinches in the second phase of the formalin test ( $p < 0.05$ ), which is reversed by 5 mg/kg naloxone and dissipated at 10 days postinfection.

using a sensitive chemiluminescent assay for quantifying  $\beta$ -galactosidase,<sup>2</sup> which showed relatively little expression in brain areas outside the amygdalar region.

#### NOCICEPTIVE RESPONSES: FORMALIN TEST

To examine the effects of enkephalin overexpression in amygdala on supraspinal nociception, the formalin test was conducted in separate groups of animals either 4 or 10 days postinfection. Formalin (50  $\mu$ L, 1%) was injected subcutaneously into the dorsal surface of the left hind paw, and the number of flinches was recorded during 5-min epochs for 60 minutes. Data were analyzed using analysis of variance (ANOVA) with repeated measures (time;  $\alpha = 0.05$ ) and post-hoc Newman-Keuls tests. A biphasic flinching response was seen, with an acute phase over 0–10 min and a tonic phase from 25–60 minutes. When compared to infection with SHZ.1, infection with SHPE caused a reduction of the flinching behavior in the second phase of the formalin test without affecting the first phase response.<sup>1</sup> As seen in FIGURE 1, the peak-responses (flinches at 35–45 min) were reduced by enkephalin overexpression in the amygdala at 4 days postinfection. This reduction in tonic-phase flinching be-



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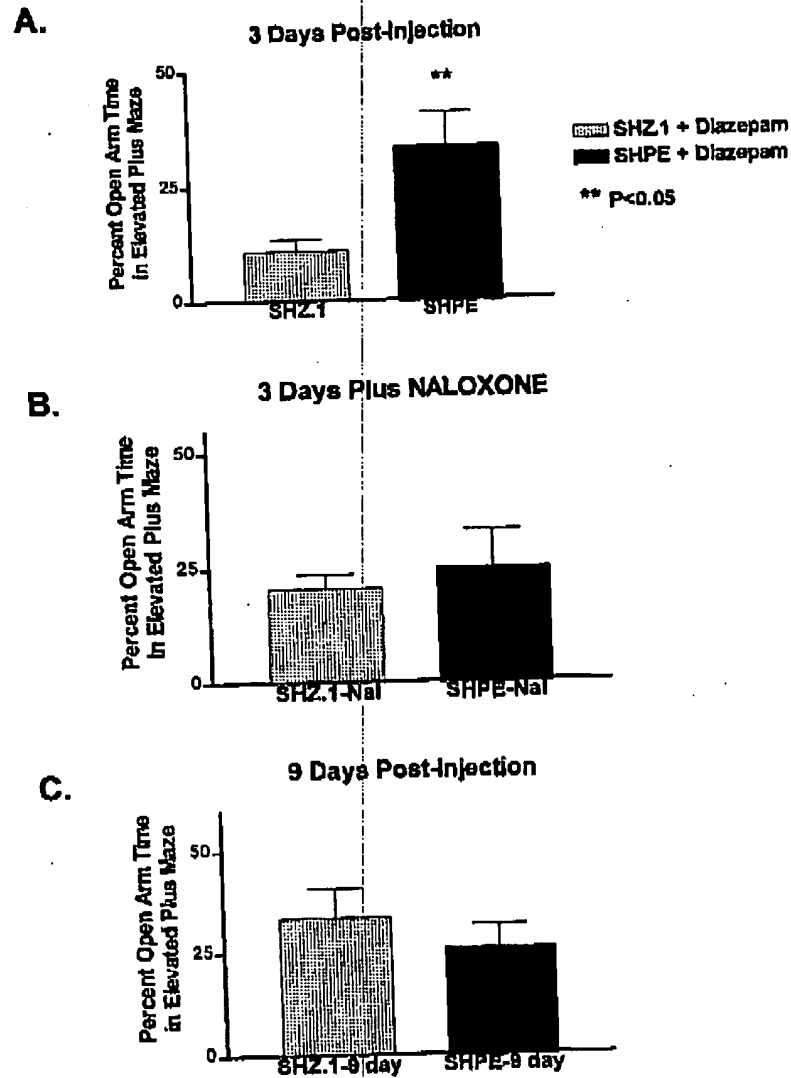


FIGURE 2. Three days after SHPE injection into the amygdala, rats show enhanced anxiolytic responses to diazepam, as shown by increased open arm time (panel A;  $p < 0.05$ ) and open arm entries (data not shown) in the elevated plus maze. These enhanced responses to diazepam were reversed by 5 mg/kg naloxone (panel B) and not apparent at 9 days postinjection (panel C).

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havior in SHPE-infected animals was reversed by the opioid antagonist naloxone hydrochloride (5 mg/kg, ip), given 10 min before formalin testing. No difference between SHZ.1 and SHPE-infected animals was seen at 10 days postinfection, suggesting that the antinociceptive effects of enkephalin overexpression had diminished by this time point.

**ANXIOLYTIC EFFECTS OF BENZODIAZEPINES:  
ELEVATED PLUS MAZE**

Anxiety levels and the effectiveness of the anxiolytic benzodiazepine agonist, diazepam, were examined at 3 days or 9 days after surgery using the elevated plus maze task.<sup>3</sup> Rats received either vehicle (10% ethanol, 40% propylene glycol, ip), or diazepam (1 mg/kg, ip) 30 min before testing, and naloxone hydrochloride (5mg/kg ip) was given simultaneously with diazepam in some rats. A reduced anxiety state was indicated by increased open arm activities, and the number of closed arm entries was used as a measure of locomotor activity. The behavioral measures were compared using *t*-tests (experiments with 2 groups) or ANOVA with post-hoc Bonferroni's multiple comparison tests.

Although SHPE infection alone did not reduce anxiety at 3 days postinjection, rats infected with SHPE exhibited a greater response to the anxiolytic effect of diazepam, when compared to rats infected with a control virus containing the lacZ gene (SHZ.1). Figure 2 shows the enhanced open arm time induced by SHPE injection in the amygdala compared with responses in SHZ.1-treated rats following diazepam administration, with no change in closed arm entries (data not shown). This enhancement of diazepam action was reversed by naloxone and correlated with preproenkephalin expression because the behavioral changes disappeared after gene expression dissipated at day 9 postinfection (Fig. 2).

**SUMMARY**

These results using herpes virus-mediated gene transfer to overexpress enkephalin in the amygdala support the role of amygdalar opioids in the anxiolytic actions of benzodiazepines and supraspinal nociception<sup>4-7</sup> (see ref. 1). These studies also demonstrate the usefulness of recombinant herpes virus in evaluating the role of single gene products within specific brain sites in pharmacological responses and complex behaviors.

**ACKNOWLEDGMENT**

This work was supported by the USC Venture Fund, the SOM Research Development Fund, and the NIDA KO2 00249 to M.A.W.

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## APPENDIX D

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# PNAS

Proceedings of the National Academy of Sciences of the United States of America [www.pnas.org](http://www.pnas.org)**Female preproenkephalin-knockout mice display altered emotional responses**

A. Ragnauth, A. Schuller, M. Morgan, J. Chan, S. Ogawa, J. Pintar, R. J. Bodnar, and D. W. Pfaff

*PNAS* 2001;98:1958-1963; originally published online Feb 6, 2001;  
doi:10.1073/pnas.041598498**This information is current as of December 2006.**

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# Female preproenkephalin-knockout mice display altered emotional responses

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Contributed by D. W. Pfaff, December 18, 2000

The endogenous opioid system has been implicated in sexual behavior, palatable intake, fear, and anxiety. The present study examined whether ovariectomized female transgenic preproenkephalin-knockout (PPEKO) mice and their wild-type and heterozygous controls displayed alterations in fear and anxiety paradigms, sucrose intake, and lordotic behavior. To examine stability of responding, three squads of the genotypes were tested across seasons over a 20-month period. In a fear-conditioning paradigm, PPEKO mice significantly increased freezing to both fear and fear + shock stimuli relative to controls. In the open field, PPEKO mice spent significantly less time and traversed significantly less distance in the center of an open field than wild-type controls. Further, PPEKO mice spent significantly less time and tended to be less active on the light side of a dark-light chamber than controls, indicating that deletion of the enkephalin gene resulted in exaggerated responses to fear or anxiety-provoking environments. These selective deficits were observed consistently across testing squads spanning 20 months and different seasons. In contrast, PPEKO mice failed to differ from corresponding controls in sucrose, chow, or water intake across a range (0.0001–20%) of sucrose concentrations and failed to differ in either lordotic or female approach to male behaviors when primed with estradiol and progesterone, thereby arguing strongly for the selectivity of a fear and anxiety deficit which was not caused by generalized and nonspecific debilitation. These transgenic data strongly suggest that opioids, and particularly enkephalin gene products, are acting naturally to inhibit fear and anxiety.

**E**ndogenous opioid systems are involved intimately in fear-related behaviors. For example, the work of Fanselow and coworkers has established an opioidergic modulation of freezing responses in rodents, which is a basic species-specific response to threatening stimuli (1–5). It would be hypothesized then that gene deletion of an endogenous enkephalin opioid peptide gene should alter the ability of the animal to respond appropriately to fear- or threat-inducing stimuli as evidenced in paradigms ostensibly measuring these responses including the open-field, dark-light transition, and conditioned shock paradigms.

Opioid agonists have been shown also to potentially stimulate intake of sweet solutions (6–12), ostensibly by increasing the hedonic value of the solution. It then would be hypothesized that loss of an endogenous enkephalin opioid peptide should blunt the proingestive actions of sucrose over a range of sucrose concentrations.

Finally, previous molecular data lead to a prediction about possible effects of an enkephalin gene knockout on estrogen-dependent lordosis behavior. Estrogen administration rapidly induces high levels of enkephalin mRNA in the ventromedial nucleus of the hypothalamus (VMH), a nerve-cell group essential for lordosis behavior (13, 14). This induction includes a transcriptional facilitation by estradiol (15–17) and is greater in females than in males (18). That the enkephalin induction might be related causally to female reproductive behavior is likely; antisense DNA directed against enkephalin mRNA and micro-injected into the VMH significantly reduced lordosis behavior

(19). Therefore, we predicted that female mice bearing enkephalin gene deletions would show lower levels of lordosis behavior compared with their wild-type (WT) controls.

## Materials and Methods

Female transgenic preproenkephalin-knockout (PPEKO) mice and their WT and heterozygous (HZ) littermates were used. The mice were bred in mixed-background C57BL/6J and 129 strains. The preproenkephalin gene was targeted by probing a murine Sw129/ReJ genomic library and isolating the enkephalin gene. The target vector was produced by subcloning two genomic DNA fragments into a pBluescript II SK-based vector (obtained from S. Potter, University of Cincinnati, Cincinnati) containing the *neo* and herpes simplex virus (HSV)-*tk* genes, both driven by the HSV thymidine kinase promoter. First, a 2.1-kb *Xba*I fragment, containing the 3' part of exon 3 and part of intron 3, was filled in and subcloned into a blunt *Hind*III site of the cassette. The resulting construct was digested with *Not*I, filled in, and then ligated to a blunt 6-kb *Sal*I-*Bgl*II fragment containing exon 1, intron 1, exon 2, and a part of intron 2.

At 5–7 weeks of age, mice were grouped by genotype and shipped from the University of Medicine and Dentistry of New Jersey in three different shipments to The Rockefeller University over a 20-month test period. These three different squads of animals began testing in the Winter of 1997, Summer of 1998, and Spring of 1999, because questions have been raised recently about the consistency of behavioral data in gene-knockout studies (20). After arrival at The Rockefeller University, all mice were housed individually in plastic cages (30 × 20 × 15 cm) throughout the entire study and maintained on a 12/12-h light-dark cycle (light off at 11 a.m.) at a constant temperature of 22°C. Mouse chow and water were available ad libitum. After approximately 10 days of acclimation, all mice were anesthetized and ovariectomized, and each was s.c. implanted with a silastic capsule (1.5-cm long) containing 17  $\beta$ -estradiol benzoate (50  $\mu$ g in 0.1 ml of sesame oil total volume). Then, 5 h before testing, female mice were primed with a s.c. injection of progesterone (500  $\mu$ g in 0.1 ml sesame oil) to ensure high sexual receptivity.

By using the  $\chi^2$  test, a significant effect of mortality was noted between PPEKO and WT mice ( $\chi^2 = 6.23$ ,  $P < 0.05$ ,  $df = 2$ ). For example, in the first two squads of mice ( $n = 22$  of each genotype), 12 PPEKO, 16 HZ, and 21 WT survived surgery. Similar differences in postsurgical recovery rates also were noted (data not shown), such that PPEKO took longer to heal after surgery than either WT or HZ mice. Testing began 3 weeks after

Abbreviations: WT, wild type; PPEKO, preproenkephalin-knockout; HZ, heterozygous; CS, conditioned stimulus; n.s., not significant.

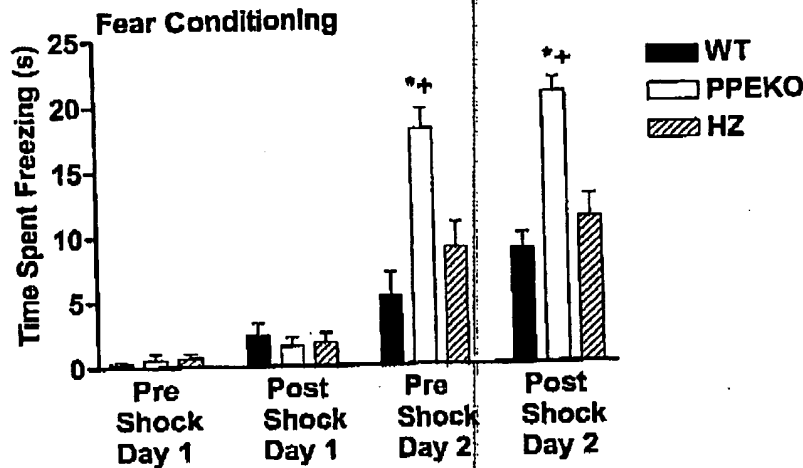
J.P., R.J.B., and D.W.P. contributed equally to this work.

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**Fig. 1.** Alterations in fear conditioning in WT mice, PPEKO mice with both alleles eliminated, and preproenkephalin HZ knockout mice with only one allele eliminated (HZ). The amount of time in a 30-sec period in which the animal exhibited freezing behavior (mean  $\pm$  SEM) was assessed in animals during an auditory stimulus before (Pre Shock) and after (Post Shock) foot shock on 2 consecutive days (Days 1 and 2). Freezing behavior observed during the first day of auditory stimulation that served as the neutral stimulus (Pre Shock, Day 1) did not differ from the first day of shock, which served as the shock stimulus (Post Shock, Day 1). The PPEKO and HZ groups displayed significantly greater freezing responses during the second day of auditory stimulation, which served as a measure of fear conditioning (Pre Shock, Day 2), and all three groups displayed significantly greater freezing responses during the second day of shock, which served as the fear + shock condition (Post Shock, Day 2) (data not shown). PPEKO mice displayed significantly greater freezing responses during the second day of auditory stimulation both before (Pre Shock, Day 2, fear condition) and after (Post Shock, Day 2, fear + shock condition) shock relative to either WT (\*) or HZ (†) mice. Significant differences were observed among genotypes [ANOVA  $F(2,28) = 22.32$ ; Tukey's  $t$  test,  $P < 0.0001$ ], across conditions [ $F(3,18) = 184.08$ ,  $P < 0.0001$ ], and for the interaction between genotypes and conditions [ $F(6,84) = 12.54$ ,  $P < 0.0001$ ]. Variability as a function of squads of testing failed to account for any significant results.

the ovariectomized mice were implanted with 17  $\beta$ -estradiol benzoate.

**Sex-Behavior Methods.** The ovariectomized females were tested for sexual behavior with sexually experienced and vigorous males (Swiss-Webster, 25–30 g) between 2 and 3 h into the dark portion of the light–dark cycle over 6 consecutive days. Testing occurred in the males' home cage in the females' home room. Each test lasted for 15 min or until the male mounted the female 17 times, whichever occurred first. Female mice were assigned randomly to a male. By using both on-site observation and videotape review, a behavioral observer blind to the genotype of the females recorded lordotic responses of the female to male mounting, intromission, and ejaculation. Female responses to male mounts or intromissions were scored as (i) totally unresponsive, kicking, rearing, boxing, or fleeing (score 0); (ii) proceptive–still posture with extension of legs (score 0.5); and (iii) receptive–lordosis posture with dorsiflexion of the vertebral column (score 1–3 using 0.5 intervals, depending on the angle of the degree of female dorsiflexion). Female responses that scored 1 or higher were considered lordotic responders and were included for the calculation of the lordosis quotient [LQ = the total number of lordosis responses/total number of mounts multiplied by 100 ( $L/M \times 100$ )]. After completion of sex-behavior testing, the silastic capsule containing 17  $\beta$ -estradiol benzoate was removed under light anesthesia, and the animals were allowed to recover for 2 weeks.

**Ingestive-Behavior Methods.** After this recovery period, mice were tested in the ingestive-behavior paradigm. Because all three squads had been separated previously and grouped by genotype, each group was divided randomly in half. The first half of each group then was assigned to a series of conditions that included 24-h daily exposure to nine ascending series of sucrose concentrations: 0% (2 days), 0.0001, 0.001, 0.01, 0.1, 1, 5, 10, 20, and

then 0% (2 days) again. The second half of each group received identical conditions except they were exposed to a descending series of sucrose concentrations. In this paradigm, all animals were provided with two preweighed bottles: one containing the particular concentration of the sucrose solution and the other containing water. Mice were trained initially with both bottles filled with water for 4–5 days before data acquisition. The positions of the bottle placements were counterbalanced across animals and were varied within animals according to an ABBA design. Fresh preweighed mouse chow was provided to the mice at the same time as they were given their water and sucrose bottles. Chow intake, adjusted for spillage, sucrose intake, and water intake were each calculated daily.

**Open-Field Methods.** Two weeks elapsed after the ingestive-behavior paradigm to minimize interactions between paradigms. All female mice were tested starting between 2 and 3 h into their dark cycle for 10 min each day on 3 consecutive days in an open-field apparatus (50-cm long  $\times$  50-cm wide  $\times$  35-cm high, clear plastic wall), which was illuminated by a 100-W white bulb suspended 1 m directly overhead. Testing occurred in their home room and a thick light-opaque curtain was used to maintain darkness and block any sounds from escaping the test area. Animals were removed directly from their cages and gently placed nose first into a specified corner of the open-field apparatus (Digiscan model RXYCM16TAO, Omnitech Electronics, Columbus, Ohio), and after release, data acquisition began. A Digiscan analyzer (DCM-8) and DIGISCAN software were used to analyze and store horizontal-activity data, which was monitored automatically by infra-red beams. Total moving time, total moving distance, moving time in the center, moving distance in the center, moving time in the margins, and moving distance in the margins were recorded for each mouse.

**Dark-Light Transition Methods.** Two weeks after the open-field testing, each of the female mice were tested starting between 2

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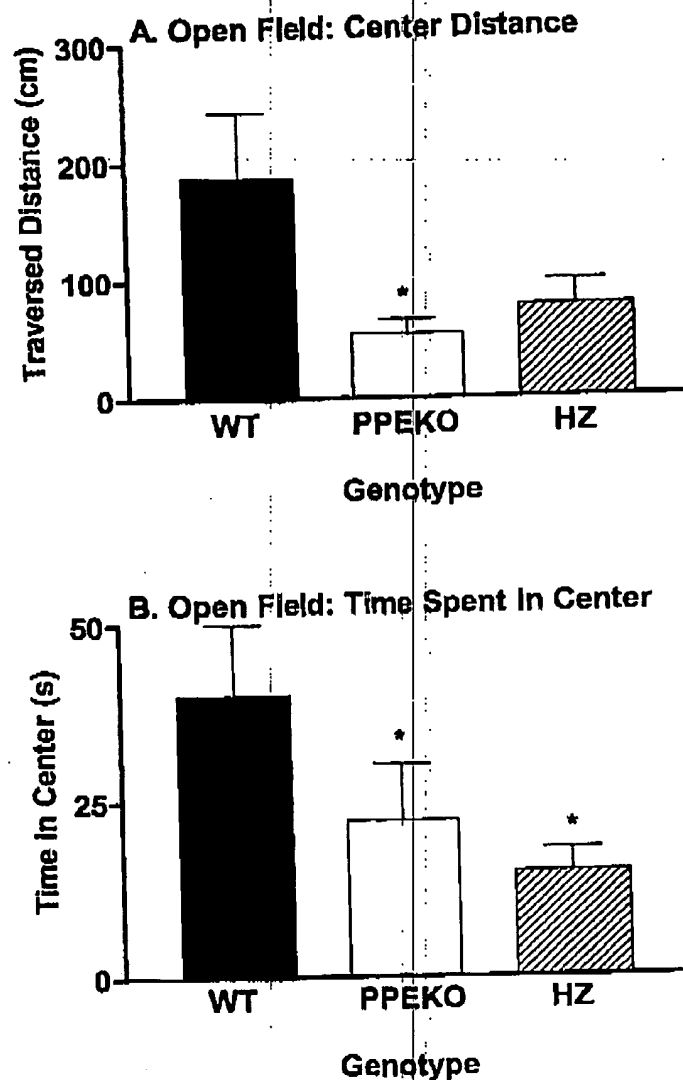


Fig. 2. Alterations (mean  $\pm$  SEM) in either the amount of distance traversed (A) or the amount of time spent (B) in the center of an open field by WT, PPEKO, and HZ mice. PPEKO mice traversed significantly less distance [ANOVA,  $F(2,28) = 5.52$ , Tukey's  $t$  test,  $P < 0.01$ ] and spent significantly less time ( $F = 3.49$ ,  $P < 0.044$ ) in the center of the open field than WT mice (\*). HZ mice also spent significantly less time in the center of the open field than WT mice. Although the second squad of animals displayed significant increases in the traversed distances [ $F(2,12) = 6.88$ ,  $P < 0.01$ ] and elapsed time [ $F(2,12) = 14.98$ ,  $P < 0.0005$ ] in the center of the open field than the first and third squads, these effects occurred consistently over all genotypes and failed to account for any significant genotype results.

and 3 h into their dark cycle for 10 min each day on 3 consecutive days in a dark-light apparatus (50-cm long  $\times$  50-cm wide  $\times$  35-cm high, clear plastic wall), with a black (light opaque) 50-cm long  $\times$  25-cm wide  $\times$  25-cm high covered-plastic box on one side (the dark side) that had an open 2-cm high  $\times$  5-cm long doorway that led to the light side of the apparatus, which was illuminated by a 100-W white bulb suspended 1 m directly overhead. Testing occurred in their home room, and a thick light-opaque curtain was used to maintain darkness and block any sounds from escaping the test area. Animals were removed directly from their cages and gently placed nose first into the doorway of the dark side of the dark-light apparatus, and after release data acquisition began. A Digiscan analyzer and DIGISCAN software were used to analyze and store horizontal-activity data, which was

monitored automatically by infra-red beams. Each mouse had the following measurements taken: total moving time and total moving distance for each compartment, and total time in dark, total time in light, total distance traveled in dark and in light, and the latency to move from the dark to the light compartment.

**Fear-Conditioning Methods.** Two weeks after the dark-light transition paradigm, conditioned-fear behavior (freezing responses) was assessed to measure aversive emotional learning in the three genotypes. Each of the female mice were tested starting between 2 and 3 h into their dark cycle. Freezing behavior was measured on an initial-acquisition day and then 24 h thereafter to measure fear responses after shock. Conditioning took place in a Plexiglas sound-attenuated chamber with a metal grid floor. Each mouse

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was placed in the test chamber and allowed to explore freely for 2 min. After this exploration, a white-noise conditioned stimulus (CS) (75-dB piezo buzzer, Radio Shack) was presented for 30 sec and coterminated with a mild (2-sec, 0.5 mA) foot shock. The mouse was removed from the chamber 1.5 min later and returned to its home cage. Then 24 h later, the mouse was placed back into the test chamber and exposed to identical testing conditions. The presence of freezing behavior was recorded during the 30 sec of auditory stimuli on each of the two days and during the 30 sec after foot shock on each of the two days. The first day of auditory stimulation served as a neutral stimulus, and the first day of shock served as the shock stimulus. The second day of auditory stimulation, acting as a CS for shock, served as a measure of fear conditioning, whereas the second day of shock served as the fear + shock condition. Then 9 days later, the CS was presented alone.

Three-way randomized block ANOVA was performed on each of the dependent variables in each of the paradigms that examined the main effects and interactions of the three experimental groups (PPEKO, HZ, and WT), the three testing squads (batches 1, 2, and 3), and the levels of the independent variable under study (e.g., sucrose concentrations in the sucrose drinking paradigm). If significant main or interaction effects were observed, Tukey-corrected post hoc comparisons were performed then to ascertain specific significant experimental effects relative to corresponding control data.

### Results

**Fear Conditioning.** In Fig. 1, it is clear that all three genotypes froze (became motionless) in response to the auditory stimulus acting as the CS for shock on day 2, as compared with day 1. Likewise, their postshock freezing was significantly greater on day 2 than on day 1. Animals did not freeze substantially to the context before the presentation of the CS on any of the test days. Notably, the PPEKO mice froze significantly more to the CS before shock on day 2 than either the WT or HZ controls (Fig. 1). PPEKO mice also displayed a significant increase in freezing after shock on day 2, both relative to freezing levels on day 1 and as compared with their WT and HZ controls (Fig. 1). These differences among groups persisted as nonsignificant trends 9 days later without intervening training (data not shown). Notably, these comparisons among experimental genotype groups were consistent across each of the three testing squads; therefore, their data were combined. Thus, in response to a signal for fear, the PPEKO animals were significantly more reactive than the control groups.

**Open-Field Assay.** The mouse's activity in specific parts of the "open-field" chamber comprises an assay of its response to a novel anxiety-provoking situation. A mouse that is anxious in response to novelty, by definition, locomotes less, and when it does move it tends to hug the margins of the open-field chamber. In contrast, a mouse that is not anxious spends more time in the center of the apparatus (away from the walls) and traverses more distance in the center of the apparatus. PPEKO mice traversed significantly less distance in the center squares of the open field (Fig. 2A) and spent significantly less time in the center of the open field (Fig. 2B) than corresponding WT controls. These differences were observed consistently in all three testing squads of the study, especially for the first and the third squads. Thus, in concert with the fear-conditioning results, the enkephalin-knockout animals appeared to display an exaggerated response to an anxiety-provoking situation.

**Dark-Light Transition Assay.** In this test, the questions "if a mouse is placed in the dark side of a standard test chamber, will it come out into the light?" and "How much time does it spend there?" are asked. A more anxious animal by definition spends more

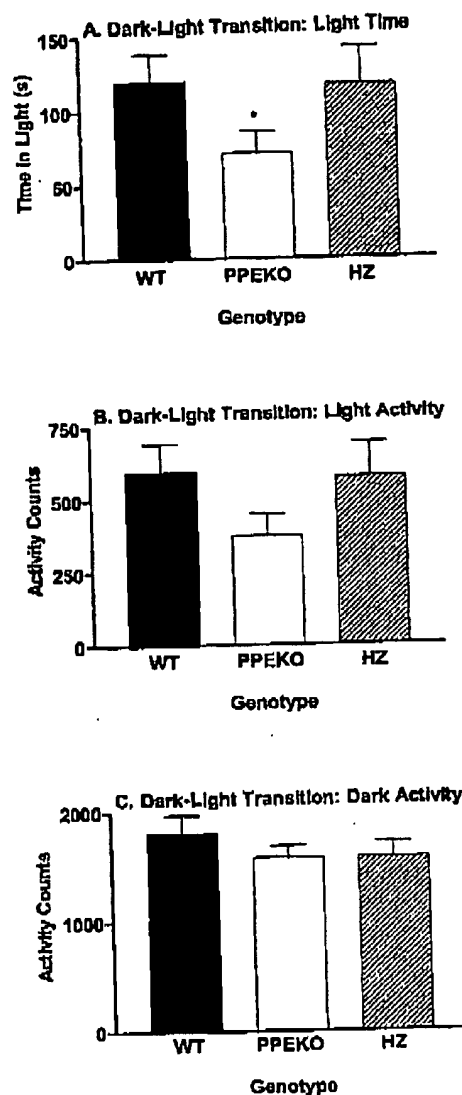


Fig. 3. Alterations (mean  $\pm$  SEM) in either the amount of time spent in the light compartment (A) or the amount of activity occurring in the light (B) and dark (C) compartments in WT, PPEKO, and HZ mice. PPEKO mice spent significantly less time [ANOVA,  $F(2,28) = 3.89$ ; Tukey's  $t$  test,  $P < 0.032$ ] in the light compartment than either WT or HZ mice (\*). The activity of the three groups in either the light ( $F = 2.50$ ,  $P < 0.10$ ) or dark ( $F = 0.44$ , not significant (n.s.)) compartments failed to differ significantly from each other. Significant differences were not observed either among squads (light time,  $F(2,12) = 2.97$ , n.s.; light activity,  $F = 1.99$ , n.s.; dark activity,  $F = 0.28$ , n.s.) or for the interaction between genotypes and squads (light time,  $F(4,24) = 2.12$ , n.s.; light activity,  $F = 2.21$ , n.s.; dark activity,  $F = 1.80$ , n.s.).

time in the dark and less time in the light. PPEKO mice spent significantly less time in the light side than either WT or HZ controls (Fig. 3A), and the knockout mice also tended to be less active once in the light side of the compartment (Fig. 3B). This latter result was not caused simply by a locomotor deficit, because activity levels of PPEKO mice on the dark side of the chamber were equivalent to the two control groups (Fig. 3C). This finding was confirmed in a separate assay of locomotor

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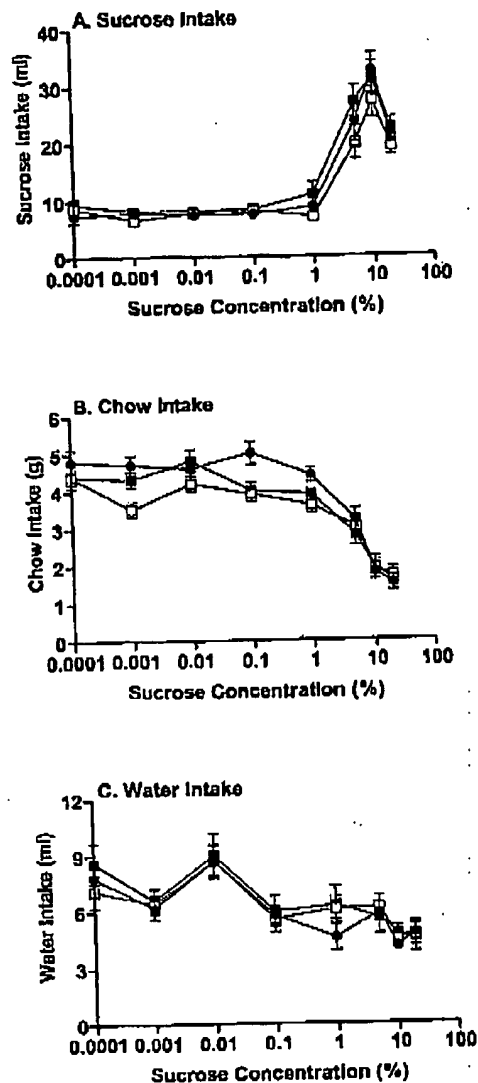


Fig. 4. Alterations (mean ± SEM) in sucrose (A), chow (B), or water (C) intake across a range (0.0001–20%) of sucrose concentrations in WT (dark circles), PPEKO (open squares), and HZ (closed squares) mice. As expected, significant increases in sucrose intake [ANOVA,  $F(8,48) = 334.38$ ; Tukey's  $t$  test,  $P < 0.0001$ ] and significant decreases in chow ( $F = 319.57$ ,  $P < 0.0001$ ) and water ( $F = 47.47$ ,  $P < 0.0001$ ) intakes were observed as a function of sucrose concentration. However, the patterns of each of the three types of intake across the sucrose concentrations failed to differ among genotypes [sucrose,  $F(2,30) = 2.76$ ,  $P < 0.07$ ; chow,  $F = 2.71$ ,  $P < 0.083$ ; water,  $F = 2.44$ , n.s.]. Although the second squad of animals consumed significantly more sucrose [ $F(2,12) = 29.42$ ,  $P < 0.0001$ ] and chow ( $F = 4.12$ ,  $P < 0.044$ ) and drank significantly less water ( $F = 27.71$ ,  $P < 0.0001$ ) than the first and third squads, these effects occurred consistently over all genotypes and failed to alter the genotype results.

capacity by using standard running wheels in the home cage that failed to show differences among genotypes (data not shown). Again, these genotype differences in the dark-light transition assay consistently occurred across all three testing squads with no significant changes observed as a function of the testing squad. Thus, as with the fear conditioning and open-field assays,

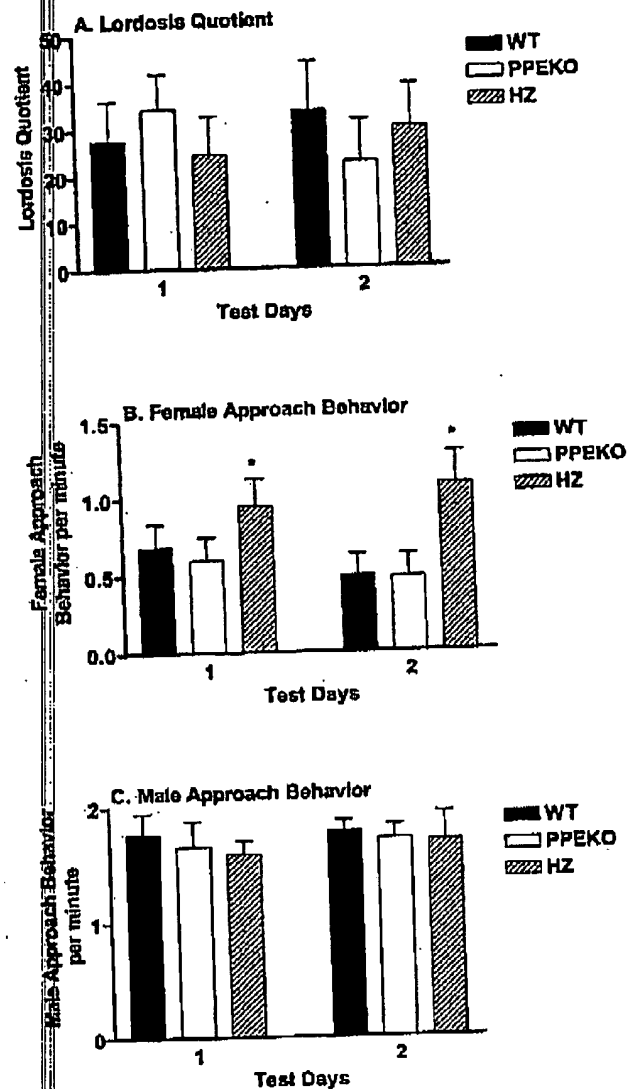


Fig. 5. Alterations (mean ± SEM) in the lordosis quotient (A) and approach behaviors of female (B) and male (C) mice in sexual encounters involving WT, PPEKO, and HZ female mice. Although the three groups failed to differ in lordotic behavior [ $F(2,30) = 0.07$ , n.s.], HZ mice produced significantly more approach behaviors (\*) than the other two groups ( $F = 4.66$ ,  $P < 0.017$ ). In contrast, male approach behavior failed to differ as a function of female genotype ( $F = 0.39$ , n.s.). Although the third squad of animals displayed significantly less lordotic behavior [ANOVA:  $F(2,12) = 22.76$ , Tukey's  $t$  test:  $P < 0.0001$ ] and the first squad of animals displayed significantly more female approach behavior ( $F = 18.86$ ,  $P < 0.0002$ ) relative to the other squads, these effects occurred consistently over all genotypes and failed to alter the genotype results.

the enkephalin-knockout mice appeared to show an exaggerated response to an anxiety-provoking situation in dark-light transition testing.

**Sucrose Intake.** When one follows the amount of sucrose intake as a function of sucrose concentration, crucial differences typically occur at the breakpoint at which sucrose intake dramatically increases. Contrary to our expectation of substantial de-

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creases in sucrose intake across concentrations in PPEKO mice, only small and nonsignificant reductions were observed in these animals at these critical 1, 5, and 10% sucrose concentrations (Fig. 4A). Moreover, the systematic decreases in chow (Fig. 4B) and water (Fig. 4C) intakes across sucrose concentrations were similar for all three genotypes. The similar pattern of these effects was noted consistently for each testing squad, despite overall decreases in sucrose intake noted in mice in the second squad relative to the first and third testing squads (data not shown). The consistency of intakes of sucrose, water, and chow in the three groups of mice suggest that nonspecific behavioral deficits were not responsible for the exaggerated responses observed in PPEKO mice in the previous three paradigms.

**Female Mating Behavior.** When measuring the lordosis quotient, significant differences failed to be observed among genotypes (Fig. 5A). Surprisingly, female HZ mice approached males more frequently than either WT or PPEKO mice (Fig. 5B). The behavior of the females apparently was not influenced by differential treatment of particular females by the male, because male approach behaviors were equivalent among genotypes (Fig. 5C). Notably, the equivalence among genotypes with respect to the lordosis quotient and male approach behavior were stable across all testing squads.

## Discussion

Across three different kinds of assays, fear conditioning, open-field activity, and dark-light transition, the enkephalin-knockout mice showed exaggerated responses to fear or anxiety-provoking environments compared with their WT and HZ controls. Surprisingly, these PPEKO mice may comprise an animal model of both heightened fear and exaggerated anxiety. These two types of behavioral responses would not necessarily co-vary, because such aspects of emotionality can be shown to have a multidimensional character (21). Exactly how and where in the central nervous system the preproenkephalin gene influences these behaviors cannot be determined from the present data. However, because viral vector-mediated preproenkephalin overexpression in the amygdala can be antinociceptive (22) and can potentiate the anxiolytic effects of benzodiazepines (23), amygdalar function may have been altered importantly by this preproenkephalin-gene knockout.

The strength of the selective deficits reported above is emphasized by the consistency of these observations over three testing squads of animals, spanning both 20 months of testing and different seasons of the year. Moreover, while this manuscript was being reviewed, we became aware of parallel experiments with  $\delta$  opioid-receptor knockout mice (24) which, as would be predicted, gave results in total agreement with the present

report. Because of these results and many further examples of other reliable bodies of data quoted in a forthcoming discussion (unpublished data) section, we believe that these data illustrating specific effects of a particular gene on a cluster of behaviors demonstrate the precision and reliability achievable with behavioral testing.

In future work, PPEKO mice conceivably could be used to ascertain whether the inhibitory actions of these opioid peptides are acting as a stimulus filter on the characteristics of open-field, dark-light, or fear-conditioning stimuli, an inhibitor of the  $\delta$  opioid-receptor-mediated substrates of fear and anxiety, and/or an inhibitory response modulator of responses to the open-field, dark-light transition, and fear-conditioning paradigms.

Compared with these predicted findings, we were surprised that the enkephalin-knockout animals failed to display either substantial decreases in sucrose consumption at critical intake concentrations (1, 5, and 10%) or reductions in lordosis behavior. However, the stable ingestive and sexual behaviors displayed by the enkephalin-knockout animals argue strongly that the anxiety differences noted were caused by a selective deficit and not by some generalized and nonspecific debilitation. Combining the two sets of positive and negative results, we now hypothesize that testing PPEKO mice for sucrose intake and for mating behavior under various stressful situations might yield important insights into how the opioid system might be engaged in modulating these motivational behaviors. Finally, the postsurgical mortality and recovery differences between PPEKO and WT mice may reflect an impaired immune system in the PPEKO animals that, when stressed, renders them particularly susceptible to challenges to their immune system and thereby may account for their increased death rate and increased recovery time.

These data provide some caveats about generalizations made in using potential gene-altering procedures. Although an antisense DNA manipulation using the antisense moiety against the preproenkephalin mRNA significantly reduced lordosis behavior if and only if it was microinjected among cells of the ventromedial nucleus of the hypothalamus (19), an effect consistent with pharmacological studies (25); a significant effect on lordosis behavior in PPEKO mice was not observed in the current study. In these and other circumstances, the differences between a site-specific and temporally specific antisense DNA manipulation, on the one hand, and a gene-knockout manipulation, on the other, show that the effect of a gene deletion can depend on exactly when and/or where the manipulation takes place.

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## APPENDIX E

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ALCOHOLISM: CLINICAL AND EXPERIMENTAL RESEARCHVol. 25, No. 9  
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## Increased Ethanol Self-Administration in $\delta$ -Opioid Receptor Knockout Mice

Amanda J. Roberts, Lisa H. Gold, Ilham Polls, Jeffrey S. McDonald, Dominique Filliol, Brigitte L. Kieffer, and George F. Koob

**Background:** The role of the  $\delta$ -opioid receptor in ethanol drinking has remained unclear despite the use of traditional pharmacological and correlational approaches. The results of several studies suggest that pharmacological blockade of these receptors results in decreases in ethanol drinking behavior, but an approximately equal number of reports have failed to observe an effect of  $\delta$ -receptor antagonism on ethanol drinking. It is clear that alternative approaches to understanding opioid-receptor involvement in ethanol drinking are needed.

**Methods:** In this study, ethanol drinking was examined in  $\delta$ -opioid receptor knockout (KO) mice by using first a two-bottle-choice test, then an operant self-administration paradigm and a second two-bottle-choice test, in that order. In addition, because KO mice were previously shown to display enhanced anxiety-like behavior relative to wild-type (WT) mice, the effect of ethanol self-administration on anxiety-like responses was determined.

**Results:**  $\delta$  KO mice initially showed no evidence of a preference for ethanol in the first two-bottle-choice drinking test; however, after an experience of operant self-administration of ethanol, a preference for ethanol developed in the second two-bottle-choice test. KO mice also showed a preference for ethanol over water and self-administered more ethanol than WT mice in the operant self-administration paradigm. The ethanol self-administered in this procedure was sufficient to reverse the innate anxiety-like response observed in this strain.

**Conclusions:**  $\delta$  KO mice showed a greater preference for ethanol and consumed more ethanol than their WT counterparts, suggesting that a decrease in  $\delta$ -receptor activity is associated with increased ethanol-drinking behavior. It is hypothesized that  $\delta$  receptors may influence ethanol self-administration at least partly through an effect of these receptors on anxiety-like behavior.

**Key Words:** Alcohol, Self-Administration, Opioid, Anxiety, Mouse.

**A**LTHOUGH IT IS generally acknowledged that the endogenous opioid system is important in ethanol drinking, the precise roles of the three receptor subtypes have remained somewhat elusive (Herz, 1997). In particular, the results of studies examining the role of  $\delta$ -opioid receptors in ethanol consumption have been inconsistent. The two principal approaches have been pharmacological (examining the effects of receptor ligands on ethanol consumption) or correlational (examining ethanol drinking with respect to  $\delta$ -receptor characteristics). Approximately

half of the published articles that used a pharmacological approach in laboratory animals support a role for  $\delta$ -opioid receptors in ethanol drinking (Franck et al., 1998; Froehlich et al., 1991; June et al., 1999; Krishnan-Sarin et al., 1995a,b; Le et al., 1993), whereas the other half support no effect of this receptor subtype in ethanol consumption (Honkanen et al., 1996; Hyttia, 1993; Middaugh et al., 2000; Stromberg et al., 1998; Williams and Woods, 1998).

$\delta$ -Receptor densities have been determined in various brain regions of rodents that differ in ethanol avidities. No differences between C57BL/6 (ethanol-drinking strain) and DBA/2 (ethanol-avoiding strain) mice in  $\delta$ -receptor gene expression were found in eight brain regions (Winkler et al., 1998). However, in another study, C57BL/6 mice were found to have higher densities of  $\delta$  receptors in the ventral tegmental area and nucleus accumbens relative to DBA/2 mice (de Waele and Gianoulakis, 1997). Depending on the ligand used for autoradiographical determination of  $\delta$ -receptor densities, either no differences (Soini et al., 1999) or higher  $\delta$ -receptor binding (De Waele et al., 1995) was observed in the alcohol-preferring (Alko alcohol; AA) relative to the alcohol-avoiding (Alko nonalcohol; ANA) rats. Clearly, the results of pharmacological and correlational studies are equivocal, and different approaches to

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clarifying the role of the  $\delta$  receptor in ethanol drinking should be pursued.

One exciting approach for studying the role of specific receptors in mediating the reinforcing effects of ethanol uses null mutant mice produced with recombinant DNA technology (Wehner and Bowers, 1995). We recently studied  $\mu$ -receptor knockout (KO) mice and found that these mice do not consume ethanol in either two-bottle-choice or operant self-administration paradigms (Roberts et al., 2000). The results of this study suggested that the  $\mu$ -opioid receptor is necessary for the rewarding effects of ethanol.  $\delta$  KO mice have been produced (Filliol et al., 2000) and provide a means of investigating the role of the  $\delta$ -opioid receptor in the actions of ethanol in a manner that complements and extends the traditional pharmacological and correlational approaches (Gold, 1996).

In this set of experiments, both two-bottle-choice ethanol drinking and operant ethanol self-administration were examined in  $\delta$  KO and wild-type (WT) mice. Two-bottle-choice tests were performed before and after operant ethanol self-administration training because it was shown previously that the extended experience with ethanol associated with operant training resulted in changes in subsequent two-bottle-choice behavior (Roberts et al., 2000). After the examination of ethanol consumption in these mice, a subset of the mice were tested in a light-dark transfer procedure to examine anxiety-like responses.

It recently has been suggested on the basis of studies with  $\mu$ - and  $\delta$ -receptor KO mice that these receptors may work in an opposing manner to regulate anxiety and mood state (Filliol et al., 2000).  $\mu$ -KO mice showed evidence of decreased anxiety-like behavior relative to WT littermates, whereas  $\delta$ -KO mice showed evidence of increased anxiety-like behavior relative to WT littermates in two tests of anxiety, the elevated plus-maze and light-dark box (Filliol et al., 2000). In this study, light-dark transfer behavior was examined in  $\delta$ -KO and WT mice immediately after ethanol drinking in the operant boxes to determine whether the anxiety-like behavior observed in KO mice could be reversed by self-administered ethanol.

## METHODS

### Subjects

The generation of  $\delta$ -opioid receptor KO mice has been described previously (Filliol et al., 2000). Briefly, gene inactivation was obtained by disruption of the first coding exon of the  $\delta$ -opioid receptor gene in 129/SV embryonic stem cells. Germline transmission occurred from the breeding of chimeric males with C57BL/6J females. Agouti mice were genotyped, and those showing germline transmission were used as founder animals to produce the F1 animals used in these experiments. A total of 14 male homozygous  $\delta$ -opioid receptor KO and 14 WT littermate mice imported from Strasbourg, France, were used in these experiments. The genetic background of these mice was a hybrid C57BL/6J  $\times$  129/SV strain. Mice were housed one to three per cage in a temperature-controlled room in which the lights were on a 12-hr light/dark cycle with lights off at 10:00 AM. After a 2-month mandatory quarantine period, experiments were initiated. Mice were 5 to 6 months old at this time.

### Two-Bottle-Choice Testing

For two-bottle-choice tests, mice were singly housed, and one bottle containing 10% ethanol and one containing water were placed on each cage. The positions of the tubes on the cage were random and approximately 6 inches apart. Mice were allowed free choice of these drinking solutions for 24-hr periods with simultaneous free access to food. Ethanol intake was calculated on the basis of bottle weights before and after each 24-hr period, and body weights were used to calculate grams per kilogram of ethanol consumed.

The first phase of this experiment involved 3 days of two-bottle-choice testing. Mice were then group housed once again and trained in the operant ethanol self-administration procedure described below. At the end of the operant testing, mice were once again singly housed and tested for two-bottle-choice ethanol drinking behavior across three 24-hr sessions.

### Operant Apparatus and Training

Six operant testing chambers outfitted for lever responding for liquid reinforcement were used in this study. Each of these clear Plexiglas (Rohm and Haas Co., Philadelphia, PA) chambers measured 14.9  $\times$  15.2  $\times$  18.3 cm and was housed within a larger exterior box (Coleman coolers; Coleman, Wichita, KS) equipped with an exhaust fan serving to ventilate the chamber and to mask background noise. One wall of each operant chamber was equipped with two levers (2.5 cm in width, 5 cm apart, and 2.5 cm from the grid floor). Between the levers there were two plastic drinking cups separated by a clear Plexiglas divider (7.5  $\times$  10 cm). A lever press required 5  $\pm$  1 g of downward force and resulted in the disruption of a photocell beam. A continuous reinforcement schedule was used, whereby a single lever press resulted in the delivery of 0.01 ml of fluid into one of the two drinking cups. Fluid delivery and recording of operant responses (photocell beam breaks) were controlled by microcomputer. Mice were tested in daily 30-min sessions, 5 days/wk.

A saccharin fading procedure used previously in mice (Roberts et al., 2000) to establish ethanol as a reinforcer was used. Both levers were available; responding to one lever resulted in the delivery of saccharin/ethanol, and responding to the other resulted in the delivery of water. The progression of saccharin fading training was as follows: 10 days of saccharin versus water, 6 days of 5% ethanol plus saccharin versus water, 4 days of 5% ethanol, 4 days of 8% ethanol plus saccharin versus water, 4 days of 8% ethanol, and 12 days of 10% ethanol plus saccharin versus water. For the final 20 days, unsweetened 10% ethanol and water were available. Throughout operant training, the lever associated with saccharin/ethanol and the lever associated with water were kept constant.

Ethanol dilutions (5, 8, and 10% w/v) were made up with 95% ethyl alcohol and water. Sodium saccharin (Sigma Chemical Co., St. Louis, MO) was added to water or the ethanol solutions to achieve a final concentration of 0.2%.

### Light-Dark Transfer Testing

The light-dark transfer procedure has been used to assess anxiety-like behavior in mice by capitalizing on the conflict between exploration of a novel environment and the avoidance of a brightly lit open field (Crawley, 1999). The apparatus was 56  $\times$  35  $\times$  35 cm (length  $\times$  width  $\times$  height), consisting of a 36  $\times$  35  $\times$  35-cm white compartment and a 20  $\times$  35  $\times$  35-cm black compartment separated by a 10  $\times$  10-cm open door. The black compartment was covered by a removable, dark Plexiglas lid. The illumination in the light compartment was 400 lux and in the dark compartment was 8 lux. Mouse behavior was recorded by using a camcorder to eliminate any disturbance caused by the presence of the investigator. Between each two mice, the apparatus was thoroughly cleaned with alcohol followed by water.

Eight WT and eight KO mice used for the ethanol drinking studies were tested in this procedure. Half of the mice of each genotype were tested for operant ethanol self-administration before light-dark transfer testing. Mice exposed to the operant ethanol self-administration paradigm

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were tested in the light-dark test immediately after the 30-min self-administration session. For testing, each mouse was placed in the white compartment, and upon entry into the dark compartment, the 5-min trial began. The number of transitions between the light and dark compartments and the time spent in each compartment were scored from the videotapes by an observer blinded to genotype and behavioral history.

Immediately after the light-dark transfer test, blood was sampled from each mouse that self-administered ethanol for blood alcohol level determinations. Approximately 40  $\mu$ l of blood was obtained by cutting 0.5 mm from the tip of each mouse's tail with a clean razor blade. Blood was collected in capillary tubes and emptied into Eppendorf tubes containing evaporated heparin and kept on ice. Samples were centrifuged and serum was decanted into fresh Eppendorf tubes. The serum was injected into an oxygen-rate alcohol analyzer (Analox Instruments, London, UK) for blood alcohol determination.

## Statistical Analyses

Preference ratios were calculated for two-bottle-choice self-administration by dividing the ethanol consumed by the total fluid consumed in the 24-hr sessions. Preference ratios were determined in the operant self-administration paradigm by dividing the number of ethanol deliveries by the total number of fluid deliveries in each 30-min session. In addition, ethanol consumption in grams per kilogram in each paradigm was calculated by using ethanol consumed and body weights. These data were analyzed by two-way analyses of variance (ANOVA) with the factors genotype (KO and WT) and sessions. In addition, two-bottle-choice preference means were analyzed by using one-sample *t* tests in which the drinking on each day was compared with a lack of preference (preference ratio of 0.5). Saccharin fading data were compared between WT and KO mice for each solution separately by using two-way ANOVA with the factors genotype (KO and WT) and session. Light-dark transfer data were analyzed by two-way ANOVA with the factors genotype (KO and WT) and treatment (ethanol self-administration and control). Significant interactions were investigated by using simple effects analyses followed by post hoc Bonferroni tests. Blood alcohol levels achieved after operant self-administration and ethanol deliveries were analyzed by two-tailed unpaired *t* tests.

## RESULTS

## Two-Bottle-Choice Testing: Before Operant Ethanol Self-Administration

Two-bottle-choice drinking results in previously ethanol-naïve mice are shown in Fig. 1. There were no significant effects of genotype, session, or genotype  $\times$  session on preference ratios. There was a significant effect of session on ethanol consumption expressed in grams per kilogram [ $F(2,52) = 5.9, p < 0.01$ ], but no other effects were statistically reliable. This session effect was explained by an overall decrease in ethanol consumption across the 3 days of testing. The preference ratio of KO mice on the first day was significantly lower than 0.5, as determined by a one-sample *t* test ( $p < 0.05$ ). Otherwise, no other preference ratio was significantly different from 0.5. These data suggest that previously ethanol-naïve WT and KO mice do not differ in ethanol consumption. Neither WT nor KO mice showed a preference for ethanol across the first 3 days of exposure, and, in fact, KO mice consumed less than half of their fluid from the ethanol bottle on the first test day ( $p < 0.05$ ).

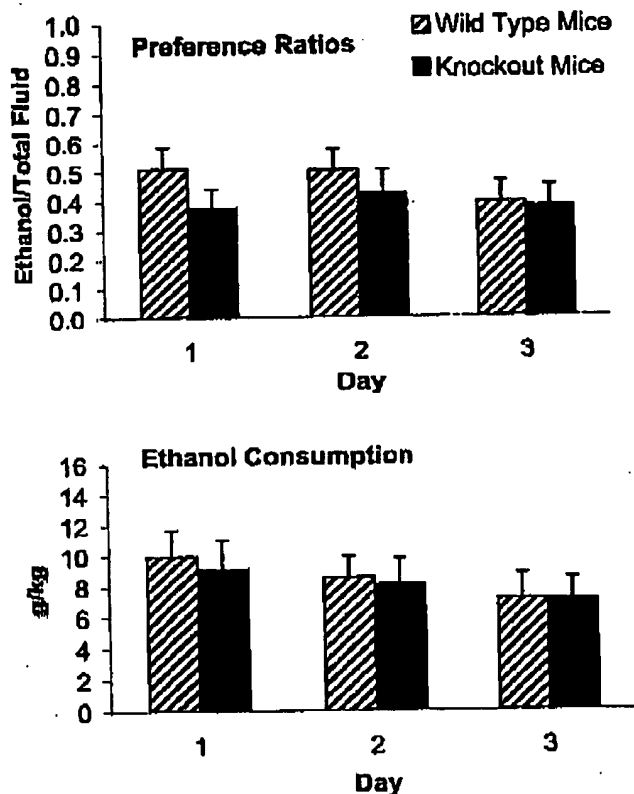


Fig. 1. Two-bottle-choice consumption of ethanol and water in ethanol-naïve 8-receptor KO and WT mice. The top panel depicts preference ratios [ethanol consumed/total fluid consumed], and the bottom panel shows ethanol consumption expressed in grams per kilogram of body weight. There were no statistically significant differences between KO and WT mice in ethanol-drinking behavior. Values shown are mean  $\pm$  SEM.

## Operant Ethanol Self-Administration

The saccharin fading operant self-administration acquisition data are shown in Fig. 2. There were no differences between WT and KO mice in responding for saccharin, 5% ethanol plus saccharin, 5% ethanol, or 8% ethanol plus saccharin. However, there was a significant effect of genotype on responding for 8% ethanol [ $F(1,26) = 5.8, p < 0.05$ ] and 10% ethanol plus saccharin [ $F(1,26) = 8.7, p < 0.01$ ]. KO mice responded more than WT mice for these solutions.

Operant self-administration across the 20 days of 10% ethanol versus water is shown in Fig. 3. There were significant effects of genotype [ $F(1,26) = 10.4, p < 0.01$ ] and session [ $F(19,494) = 4.1, p < 0.001$ ] on preference ratios. There also were statistically reliable effects of genotype [ $F(1,26) = 6.4, p < 0.05$ ] and session [ $F(19,494) = 3.2, p < 0.001$ ] on ethanol consumption in grams per kilogram. The genotype effects were due to greater preference and greater overall ethanol consumption by KO relative to WT mice. The session effects were due to slight increases in ethanol self-administration across the 20 days in both

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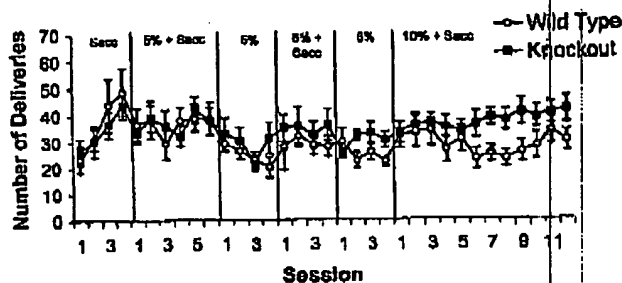


Fig. 2. Acquisition of operant ethanol self-administration by using a saccharin fading procedure. The progression of saccharin (Sacc) fading training was as follows: 10 days of saccharin versus water (the final 4 days are shown), 6 days of 5% ethanol and saccharin versus water, 4 days of 5% ethanol, 4 days of 8% ethanol and saccharin versus water, 4 days of 8% ethanol, and 12 days of 10% ethanol and saccharin versus water. There was a divergence between KO and WT mice beginning when 8% ethanol was introduced. Values shown are mean  $\pm$  SEM.

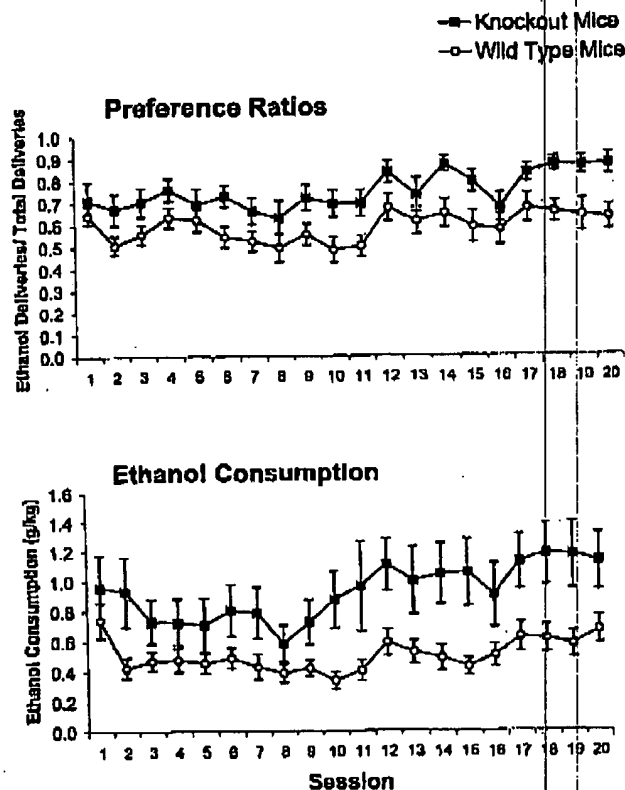


Fig. 3. Operant self-administration of 10% ethanol and water in  $\delta$ -receptor KO and WT mice. The top panel depicts preference ratios (ethanol deliveries/total fluid deliveries), and the bottom panel shows ethanol consumption in grams per kilogram of body weight. KO mice displayed greater ethanol preference and increased ethanol consumption relative to WT mice. Values shown are mean  $\pm$  SEM.

groups. One-sample  $t$  tests comparing each preference ratio mean to a ratio of 0.5 revealed a significant preference for ethanol over water in KO mice in 19 of the 20 sessions ( $p < 0.05$ ). In contrast, WT mice showed a significant prefer-

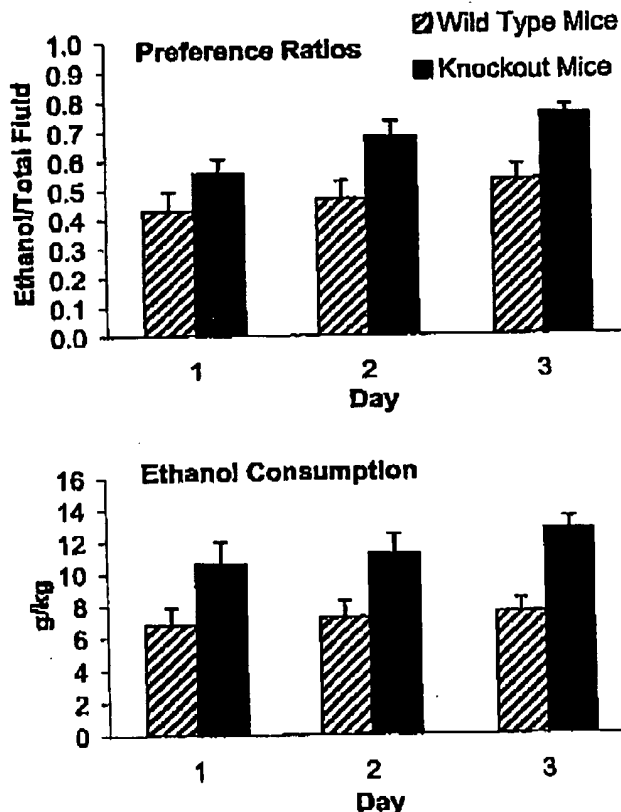


Fig. 4. Two-bottle-choice consumption of ethanol and water in ethanol-experienced  $\delta$ -receptor KO and WT mice. The top panel depicts preference ratios (ethanol consumed/total fluid consumed), and the bottom panel shows ethanol consumption expressed in grams per kilogram of body weight. KO mice displayed greater preference and increased ethanol consumption relative to WT mice. Values shown are mean  $\pm$  SEM.

ence for ethanol over water in only 8 of the 20 sessions. The results of this experiment suggest that deletion of the  $\delta$ -opioid receptor is associated with increased ethanol self-administration.

#### Two-Bottle-Choice Testing: After Operant Ethanol Self-Administration

Two-bottle-choice drinking results in ethanol-experienced mice are shown in Fig. 4. There were significant effects of genotype [ $F(1,26) = 8.6, p < 0.01$ ] and session [ $F(2,52) = 9.3, p < 0.001$ ] on preference ratios. There also was a statistically reliable effect of genotype [ $F(1,26) = 9.9, p < 0.01$ ] on ethanol consumption in grams per kilogram. KO mice displayed greater preference for ethanol and greater ethanol consumption than WT mice. Indeed, preference ratios of KO mice were significantly greater than 0.5 on the second and third test days ( $p < 0.01$ ), whereas WT mice did not show a preference in two-bottle-choice tests. The results of this experiment confirm those of the operant self-administration paradigm and



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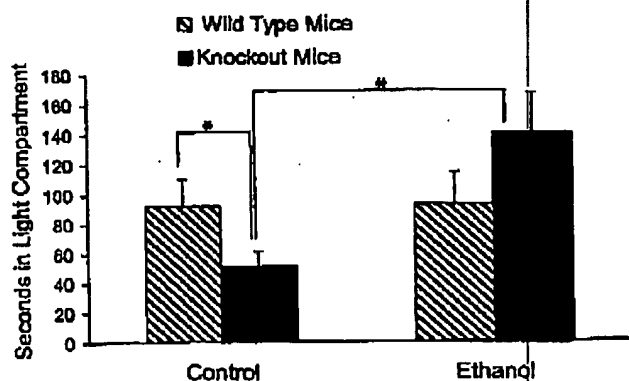


Fig. 5. Results of light-dark transfer testing, showing the time (sec) spent in the light compartment (out of a total of 300 sec) in  $\delta$ -receptor KO and WT mice. \*Statistical significance at the  $p < 0.05$  level. Values shown are mean  $\pm$  SEM.

Table 1. Ethanol Responded for by Mice (g/kg) and Corresponding Blood Alcohol Levels of Mice Allowed to Self-Administer in the Operant Chambers Before Light-Dark Transfer Testing

Mice	Ethanol deliveries (g/kg)	Blood alcohol levels (mg/100 ml)*
Wild-type mice (n = 4)	0.54 $\pm$ 0.18	5.0 $\pm$ 2.0
Knockout mice (n = 4)	1.52 $\pm$ 0.50	29.5 $\pm$ 9.4

\* Blood was sampled immediately after light-dark transfer testing (5 min after the end of the 30-min operant self-administration session).

suggest that a lack of  $\delta$ -opioid receptor activity is associated with an increase in ethanol consumption after initial exposure.

#### Light-Dark Transfer Testing

The amount of time spent in the light compartment and the numbers of transitions between the light and dark compartments were analyzed in  $\delta$ -KO and WT mice. Subjects allowed access to operant ethanol self-administration were compared with those that were not. There were no effects of genotype or treatment on number of transitions (data not shown). Figure 5 shows the time spent in the light compartment (out of a total of 300 sec) for each group. The interaction between genotype and treatment was statistically reliable [ $F(1,12) = 4.85, p < 0.05$ ]. This was accounted for both by a genotype difference under baseline conditions ( $p < 0.05$ ) and by a difference in KO mice allowed to self-administer ethanol versus those not allowed access to ethanol ( $p < 0.05$ ). These results suggest that self-administered ethanol can reverse the innate anxiety-like behavior present in  $\delta$ -opioid receptor KO mice.

Ethanol deliveries and the corresponding blood alcohol levels of the mice allowed access to operant self-administration before light-dark transfer testing are shown in Table 1. Although there was a trend toward a difference between WT and KO mice in ethanol responded for, it did not reach significance ( $t = 1.9, p = 0.09$ ). KO mice did, however, achieve significantly higher blood alcohol levels than WT mice ( $t = 2.5, p = 0.04$ ). The blood alcohol levels

of KO mice are in the range associated with mild, but significant, behavioral and pharmacological activity.

#### DISCUSSION

The results of these experiments indicated that  $\delta$ -opioid receptor KO mice developed a preference for ethanol during operant ethanol self-administration training. Operant responding in WT and KO mice diverged once the ethanol concentration was 8%. It is not clear whether the saccharin fading procedure was critical in this divergence or whether extended two-bottle-choice experience would have resulted in the same result. The delayed preference in KO mice may be related to their increased level of anxiety-like behavior (see below). In the initial two-bottle-choice test, both WT and KO mice consumed ethanol levels intermediate to those consumed by C57BL/6J (10 g/kg/day) and 129/J (6 g/kg/day) mice (Belknap et al., 1993). This is consistent with the mixed C57BL/6  $\times$  129 genetic background of these mice. Ethanol consumption of KO mice increased to approximately 13 g/kg/day, whereas the amount of ethanol consumed by WT mice stayed fairly constant at approximately 7 g/kg/day. The amount of ethanol consumed by KO mice was quite substantial and, as shown in a subset of mice self-administering ethanol in the operant paradigm, resulted in detectable blood alcohol levels (Table 1). The ethanol intake of KO mice in the two-bottle-choice tests that followed operant self-administration training was equivalent to that observed in the selectively bred high-alcohol preference mice (Graham et al., 1999).

This increased ethanol consumption by  $\delta$ -receptor KO mice suggests that the  $\delta$  receptor functions in a manner opposite to the  $\mu$  receptor and may actually inhibit the reinforcing effects of ethanol. This finding is somewhat surprising and is not consistent with several published studies that suggest that the  $\delta$ -opioid receptor has a facilitatory role in ethanol reinforcement (Franck et al., 1998; Froehlich et al., 1991; Hyytia and Kisanmaa, 2001; June et al., 1999; Krishnan-Sarin et al., 1995a,b; Le et al., 1993). However, there are several issues related to the pharmacological approaches commonly used and the potential role of the  $\delta$ -opioid receptor in anxiety that may help to explain our findings.

Pharmacological studies may be compromised by the pharmacokinetics of the antagonist used, a lack of selectivity of the agents available for the  $\delta$  receptor, or potential partial agonist effects in vivo of compounds shown to act as antagonists in vitro. For example, the  $\delta$ -receptor antagonist ICI 174864 was shown to decrease ethanol drinking when administered peripherally (Franck et al., 1998; Froehlich et al., 1991; Krishnan-Sarin et al., 1995a). However, it is not clear that this was an effect of central  $\delta$  receptors, because it is likely that a limited quantity of this antagonist entered the brain due to its peptide structure (Takemori and Portoghese, 1992). Intracerebroventricular administration of

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ICI 174864 had no effect on ethanol consumption (Hyytiä, 1993). In addition, ICI 174864 has relatively low affinity for  $\delta$  receptors (Corbett et al., 1993; Raynor et al., 1994). Decreases in ethanol consumption after administration of the nonpeptide  $\delta$ -receptor antagonist naltrindole have been reported (Hyytiä and Kiianmaa, 2001; Krishnan-Sarin et al., 1995a; Le et al., 1993); however, other studies found no effect of this compound on ethanol self-administration (Honkanen et al., 1996; Middaugh et al., 2000; Stromberg et al., 1998; Williams and Woods, 1998). It has been suggested that moderate doses of naltrindole (in the range used in these previous experiments) may produce some partial agonist activity (Jackson et al., 1989), and this compound has low selectivity for  $\delta$  receptors (Corbett et al., 1993). Finally, another  $\delta$ -receptor antagonist, naltriben, has been shown to decrease ethanol drinking in both free-choice (Krishnan-Sarin et al., 1995b) and operant self-administration (June et al., 1999) paradigms. Thus, investigations of  $\delta$ -receptor involvement in ethanol consumption that use the existing pharmacological approaches have produced mixed effects.

In support of a previous study (Filliol et al., 2000),  $\delta$ -KO mice in this study showed a greater anxiety-like response than WT mice in the light-dark transfer test. This anxiety-like behavior was less robust than in the previously published study (there was no effect of genotype on number of transitions between the light and dark compartments), perhaps due to the extensive handling, exposure to ethanol, or both that mice in the self-administration studies had experienced. However, this anxiety-like behavior of KO mice was attenuated by self-administered ethanol. This is a very important finding because it suggests that one role of the  $\delta$  receptor may be in mediating the interaction between anxiety states and ethanol consumption. In fact, the initial aversion to ethanol by  $\delta$  KO mice may be related to the increased anxiety-like behavior exhibited by this line. It has been shown that stressor exposure can decrease the consumption of a novel quinine solution (Job and Barnes, 1995). Exposure to ethanol during the saccharin fading procedure may have allowed the mice to become familiar with ethanol and even experience its anxiolytic effects. Thus, the increased anxiety associated with the lack of the  $\delta$  receptor may be related to the development of a preference for ethanol in this strain.

This potential interaction among the  $\delta$ -opioid receptor, anxiety state, and ethanol consumption may help to explain the inconsistencies in the literature regarding the role of  $\delta$ -opioid receptors in ethanol consumption. The fact that  $\delta$ -receptor KO mice show increased anxiety-like behavior suggests that decreases in  $\delta$ -receptor activity may be associated with an increased anxiety state and, conversely, that increases in  $\delta$  activity may be associated with decreased anxiety-like behaviors in some cases. Both enkephalin (Konig et al., 1996) and preproenkephalin (Ragnoult et al., 2001) KO mice showed enhanced anxiety-like responses relative to their WT counterparts with several behavioral

tests. Increases in enkephalin levels in the amygdala potentiated the anxiolytic-like effects of diazepam on the elevated plus maze (Kang et al., 2000). In addition, a  $\delta$  agonist and an inhibitor of enkephalin inactivation have been shown to possess antidepressant-like activity in the rat learned-helplessness model that involves exposing the animal to stressful stimuli (Tejedor-Real et al., 1998). Thus,  $\delta$  receptors may be involved in mediating behavioral responses to stress. Stressor exposure has been shown to increase, decrease, or have no effect on ethanol consumption (Ulm et al., 1995).

Differences in the degree to which stress and anxiety states influence ethanol consumption across animal species and strains and the various drinking paradigms used may partly explain the variety in  $\delta$ -receptor effects found in the literature. For example, Sardinian alcohol-preferring (sP) rats have reduced enkephalin messenger RNA expression in the caudate putamen (Fadda et al., 1999) and show increased anxiety-like behavior relative to Sardinian alcohol-nonpreferring rats (Colombo et al., 1995). This deficit in enkephalin expression, as well as the anxiety-like behavior, was partially reversed in sP rats allowed access to ethanol in a free-choice situation for 30 days. The AA rats have decreased enkephalin levels in the nucleus accumbens which are increased after free-choice ethanol drinking (Nylander et al., 1994). It is interesting that  $\delta$ -antagonist administration in AA rats had no effect on ethanol consumption (Honkanen et al., 1996; Hyytiä, 1993). However, these rats showed decreased anxiety-like behaviors relative to ANA rats (Moller et al., 1997). Although a possible relationship among  $\delta$ -receptor activity, anxiety, and ethanol consumption is intriguing, it is likely to be complex.

Another potential issue regarding the role of  $\delta$ -opioid receptors in ethanol consumption and the inconsistencies in published reports is that  $\delta$  receptors, via their interaction with the mesolimbic dopamine system, may influence ethanol drinking under certain conditions (Cowen and Lawrence, 1999). It has been shown that  $\delta$ -antagonist administration blocked the increase in dopamine in the nucleus accumbens after a peripheral injection of ethanol (Acquas et al., 1993). Thus, it seems that  $\delta$  receptors may be involved in the increased activity of the mesolimbic dopamine system produced by ethanol. However, there is evidence that this dopamine system, although possibly important in the acquisition of ethanol self-administration or in situations of excessive drinking, may not be critical for its maintenance. Destruction of dopamine neurons with 6-hydroxydopamine has been shown to have no major effect on established ethanol self-administration (Rassnick et al., 1993). Perhaps under certain conditions (i.e., certain rodent strains or lines),  $\delta$ -opioid receptor/dopamine system interactions continue to be important in ethanol drinking behavior.

By using an approach similar to this one, it was recently shown that  $\mu$ -receptor KO mice do not self-administer ethanol. The WT comparison mice for both the  $\mu$ - and  $\delta$ -KO lines (both mixed C57BL/6Ola  $\times$  129/Sv mice)

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showed similar ethanol consumptions in the two studies (0.5–0.6 g/kg in the operant procedure and 8–10 g/kg/day in the bottle procedure). In contrast,  $\mu$ -KO mice responded for approximately 0.2 g/kg ethanol in the operant procedure and 4 g/kg/day in the bottle drinking procedure. This is comparable to what has been found in alcohol-avoiding DBA/2J mice (Belknap et al., 1993; Risinger et al., 1998). It is interesting that  $\mu$ -KO mice have displayed decreased anxiety-like behavior compared with WT mice (Filliol et al., 2000).

The opposing phenotypes of  $\mu$ - and  $\delta$ -KO mice with regard to ethanol self-administration suggest that a balance between  $\mu$  and  $\delta$  activities may modulate ethanol drinking behavior. For example,  $\mu$  receptor activation by ethanol-induced increases in  $\beta$ -endorphin may support a continuation of ethanol consumption (Gianoulakis, 1998). In contrast, low levels of enkephalin (Blum et al., 1982) and enhanced enkephalinase activity (George et al., 1991), presumably associated with decreased  $\delta$ -receptor activity, are associated with increased ethanol drinking. However, an enkephalinase inhibitor, which would increase  $\delta$ -receptor activity, was also shown to increase ethanol intake (Froehlich et al., 1991). It is clear that the role of the  $\delta$ -opioid receptor in ethanol consumption is complex and may not be unitary but may perhaps involve both interactions with the dopaminergic reward pathway and anxiety mechanisms.

## ACKNOWLEDGMENTS

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## APPENDIX F

Science Blog -- Novel molecule blocks pain receptor system

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# Science Blog

BlogAds

Wicked Lasers: Power to Burn

1999

From: American Chemical Society

Novel molecule blocks pain receptor system

*Discovery May Lead to New Treatments for Pain*

Researchers with Banyu Pharmaceutical Co. in Japan have designed a synthetic molecule that can block a molecular pathway, allowing researchers a closer look at what makes some people less sensitive to pain.

This finding will appear in the Dec. 16 issue of the peer-reviewed Journal of Medicinal Chemistry, published by the American Chemical Society, the world's largest scientific society. The article was initially published Nov. 19 on the journal's web site.

Scientists have been studying a nerve receptor called the "opiod receptor-like 1" (ORL-1), which is widely distributed throughout the central nervous system. Like other opiod receptors, ORL-1 was believed to play a key role in pain regulation. However, the natural compounds that activate ORL-1 are different than those that activate the opiod receptors, the researchers say.

In 1995, scientists found a novel hormone, called nociceptin

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or orphanin FQ, that binds to the ORL-1 receptor. They theorized that blocking the hormone may make a person less sensitive to pain. It has been difficult to test this theory because researchers have lacked an agent to block the hormone-receptor system so its functions could be observed and tested.

Now, the Japanese researchers believe they have found that agent. While there are many different ways to prevent pain, this discovery represents a new avenue for pain research and could be key to development of new and improved drugs to treat pain, says Yoshikazu Iwasawa, Ph.D., research director at Banyu Pharmaceutical Co.

Studies in mice suggest that the ORL-1 receptor and its corresponding hormones may also play important roles in anxiety, learning and memory and other neurological responses. Further understanding of the roles of this hormone-receptor system may lead to drugs not just for pain, but for a variety of neurological disorders, Dr. Iwasawa and his associates predict.

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## APPENDIX G



Probing the role of the delta opioid receptor in alcohol consumption

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Alcoholism: Clinical & Experimental Research

## Probing the role of the delta opioid receptor in alcohol consumption

- The body's endogenous opioid system has three classes of opioid receptors: mu, delta, and kappa.
- Previous research showed that mice lacking the mu opioid receptor do not drink alcohol.
- A new study shows that mice lacking the delta opioid receptor drink more alcohol.
- The delta opioid receptor may also play a mediating role between stress and alcohol consumption.

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The body's endogenous opioid system has traditionally been linked with peptides such as enkephalins and endorphins, which influence the brain's reward pathway to act as the body's natural response to pain. A study in the September issue of *Alcoholism: Clinical & Experimental Research* has found that the endogenous opioid system may also be important for the reinforcing properties of alcohol. Researchers discovered that "knocking out" the delta opioid receptor led to an increased state of anxiety as well as an increase in drinking.

"There are three classes of opioid receptor currently recognized," said Amanda J. Roberts, assistant professor of neuropharmacology at The Scripps Research Institute and lead author of the study. "They are the mu, delta, and kappa receptors. We had previously shown that mice lacking the mu opioid receptor do not drink alcohol under several different experimental conditions." For the current study, Roberts and her colleagues used mice produced by co-author Brigitte L. Kieffer in France that had been genetically modified by having their delta receptor "knocked out."

"After becoming familiar with alcohol, mice lacking the delta receptor consumed more alcohol than their genetically intact counterparts (wild type mice) did," said Roberts, "suggesting that a decrease in delta receptor activity is associated with an increase in alcohol drinking behavior. This is a surprising finding as it suggests that, at least under certain conditions, the mu and delta receptors may act in an opposing manner to regulate alcohol consumption."

In addition to the endogenous opioid system's influence on the brain's reward pathway, it also plays an important role in the body's stress response. Alcohol researchers believe that stress and anxiety are important components of alcohol consumption. In fact, stress reduction is one of the most commonly reported psychosocial benefits of drinking alcohol. Another finding of Roberts' study supports a potential link among the endogenous opioid system, stress and alcohol consumption. The delta receptor knockout (KO) mice in this experiment exhibited increased anxiety prior to drinking and, in fact, seemed to use alcohol for its anxiolytic or calming effects.

"This suggests that the delta receptor," said Roberts, "while perhaps being important in directly modulating the activity of the brain's reward pathway, also may be a key player in mediating the link between stress and alcohol consumption."

According to Tamara Phillips, professor of behavioral

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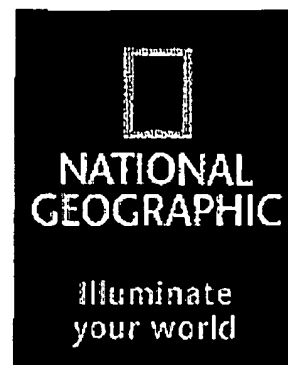
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neuroscience at Oregon Health & Science University and the Portland VA Medical Center, the study's findings also have ramifications for those alcoholism treatment strategies that utilize opiate antagonists. Opiates are drugs derived from opium - like heroin and morphine - that act like chemicals the brain produces naturally, called endogenous (from within) opioids, which stimulate pleasurable feelings and suppress pain. Medications known as opiate antagonists bind with the brain's receptors for endogenous opioids, thus blocking the desired effects of heroin and similar drugs while having no effect themselves. Although alcohol is not an opiate-like substance, opiate antagonists like naltrexone seem to block some of alcohol's rewarding effects.

"Drugs of abuse like alcohol," explained Phillips, "appear to activate some of the same brain neurochemical pathways as those activated by natural rewards such as food, water, sweets and sex. A key neurochemical is dopamine. Dopamine pathways play a well-documented role in alcohol reward and reinforcement. Opioids are known to moderate the activity of dopamine pathways, and it is possible that alcohol addiction is partly associated with alterations in opiate receptor-mediated processes. Animal and human studies documenting reductions in alcohol consumption by treatment with naltrexone, an opiate receptor antagonist drug, ultimately led to its clinical utilization for the treatment of alcoholism." Phillips added that although naltrexone is widely used in conjunction with clinical counseling, its success has been limited.

"Because this drug influences all three of the known opioid receptor subtypes, mu, delta and kappa," she said, "a worthwhile endeavor is to examine the specific roles that each of the opiate receptor subtypes might play in alcohol addiction. Naltrexone has a greater tendency to interact with mu than with delta and kappa opiate receptors. It is possible that its success in alcoholism treatment is associated with its relative affinities for these receptor subtypes, and that a better treatment agent could be developed. This study, for example, shows the importance of the delta receptor in influencing voluntary alcohol consumption."

Roberts and her colleagues plan to continue with their examination of the endogenous opioid system. They will more closely examine the brain regions and pathways responsible for the role of the mu and delta opioid receptors in alcohol's rewarding effects, as well as what role(s) the endogenous opioid system may play in addiction and relapse.



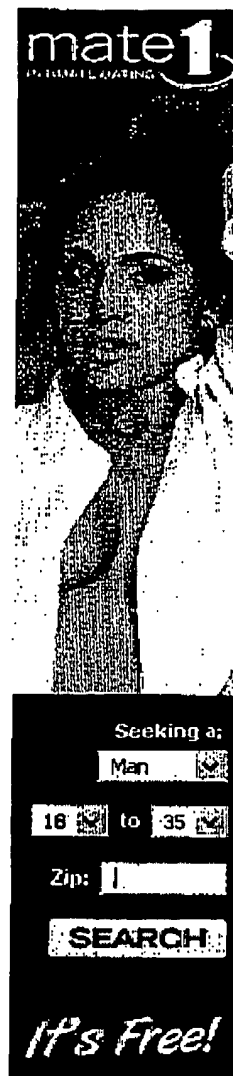
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Co-authors of the *Alcoholism: Clinical & Experimental Research* paper included: Lisa H. Gold of Neurobiology, Pharmacia Corporation in Kalamazoo, MI; Ilham Polis, Jeffrey S. McDonald, and George F. Koob of the Department of Neuropharmacology at The Scripps Research Institute; and Dominique Filliol and Brigitte L. Kieffer of ESBS Université Louis Pasteur in Strasbourg, France. The study was funded by the National Institute on Alcohol Abuse and Alcoholism, and by the Mission Interministerielle de Lutte contre la Drogue at la Toxicomanie at the Centre National de la Recherche Scientifique in France.



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Patent App No: 01533764

## APPENDIX H

DBGET Result: OMIM 165195

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*Portent App No: 10/533764***Database: OMIM**  
**Entry: 165195**

MIM Entry: 165195

Title:

\*165195 OPIOID RECEPTOR, DELTA-1; OPRD1  
;;OPRD

Text:

Bzdega et al. (1993) cloned the delta opiate receptor gene from a mouse neuroblastoma-rat glioma hybrid cell line. The clone they isolated was apparently identical to those reported by others (e.g., Evans et al., 1992). They found full-length transcripts of the gene in mouse brain but in no other tissues examined. Within the brain the gene was expressed at low levels in many regions, but transcripts were found in particularly large amounts in the anterior pituitary and pineal glands. Since these tissues are located outside the blood-brain barrier, opioid peptides easily can reach receptors in these areas from the blood. The gene, which was present in single copy and was symbolized Nbor for 'neuroblastoma opiate receptor,' was mapped to the distal region of mouse chromosome 4 by linkage studies. It was found to lie between Lck and Gnb-1. The human homologs of these 2 genes, LCK (153390) and GNB1 (139380), are located on human chromosome 1p; thus, the human gene for delta-opiate receptor is probably in this region. (GNB1 is mapped to 1pter-p31.2; LCK is mapped to 1p35-p32.) Kaufman et al. (1994) reported linkage relationships of Oprd1 on mouse chromosome 4 and stated that the human homolog had been mapped to 1p by in situ hybridization. Befort et al. (1994) assigned the OPRD1 gene to 1p36.1-p34.3 by isotopic in situ hybridization and the homologous gene to mouse chromosome 4 by the same method.

Jordan and Devi (1999) provided biochemical and pharmacologic evidence for the heterodimerization of 2 fully functional opioid receptors, kappa (OPRK1; 165196) and delta. This results in a new receptor that exhibits ligand binding and functional properties that are distinct from those of either receptor. Furthermore, the kappa-delta heterodimer synergistically binds highly selective agonists and potentiates signal transduction.

Mayer et al. (2000) noted that the delta-opioid receptor gene contains 3 exons encoding a 7-transmembrane, G protein-coupled receptor. By RT-PCR screening of melanoma cell lines for the presence of delta-opioid receptor mRNA, Mayer et al. (2000) detected a 623-bp product in addition to the expected 773-bp product. Ligand binding studies confirmed the presence of the delta-opioid receptors on pigment cells at the expected binding capacity but at somewhat lower density than expected. Further RT-PCR screening determined that the normal receptor is present on all normal and malignant pigment and neuronal cells, whereas the short form is found exclusively in the tumors. Sequence analysis indicated that the short receptor is not encoded by the genome but results from mRNA processing and a deletion, apparently by a transposon mechanism, of 144 bp within the third exon. This region corresponds to the third cytoplasmic domain of the receptor molecule.

Pharmacologic and electrophysiologic evidence indicates that opioid receptors are involved in the mechanism of heroin dependence. Thus, opioid receptors are appropriate candidate genes for case-control association studies of heroin dependence. To test the hypothesis that OPRD1 or a closely linked gene is associated with heroin dependence, Xu et al. (2002) used 5-prime nuclease assays to genotype 2 OPRD1 SNPs in 450 Chinese heroin dependent patients and 304 unaffected controls from the same population. In addition, 5 SNPs distributed in 4 other genes (ADH2, 103720; ALDH2, 100650; OPRM1, 600018; and DRD1, 126449) were used as genomic control loci to test the case and control populations for

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stratification bias. One of the SNPs, 80G, was absent from both Chinese opioid dependence patients and controls; genotype and allele frequencies at the other OPRD1 SNP, 921T-C, were not significantly different.

Whistler et al. (2002) identified a G protein-coupled receptor-associated sorting protein, GASP (300417), that interacts with the cytoplasmic tail of OPRD1 and appears to modulate OPRD1 recycling and trafficking to lysosomes. Opioid peptide activation of HEK293 cells transfected with GASP resulted in rapid endocytosis and proteolysis of OPRD1. Using several binding assays with truncated GASP proteins, Whistler et al. (2002) determined that the C-terminal portion of GASP binds specifically to the OPRD1 tail.

#### ANIMAL MODEL

Filliol et al. (2000) generated Oprd1-deficient mice and compared the behavioral responses of mice lacking Oprd1, Oprm, and Oprk1 in several models of anxiety and depression. Their data showed no detectable phenotype in Oprk1 -/- mutants, suggesting that kappa-receptors do not have a role in this aspect of opioid function. Opposing phenotypes in Oprm -/- and Oprd1 -/- mutants contrasted with the classic notion of similar activities of mu- and delta-receptors. Anxiogenic- and depressive-like responses in Oprd1 -/- mice indicated that delta-receptor activity contributes to improvement of mood states. Filliol et al. (2000) concluded that the Oprd1-encoded receptor, which has been proposed to be a promising target for the clinical management of pain, should also be considered in the treatment of drug addiction and other mood-related disorders.

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## APPENDIX I

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1: Theraple. 1992 Nov;47(6):531-9.

[Study of induced effects by selective CCKB agonists cholecystokinin in the nociception and behavior in rodents]

[Article in French]

Dauge V, Derrien M, Durieux C, Noble F, Corringer PJ, Roques BP.

Departement de Pharmacochimie Molculaire et Structurale, U 266 INSERM, Paris.

Potent and selective CCK-B agonists with good bioavailability have been designed by modifying the natural CCK-8 peptide. Thus, BC 264 [Boc-Tyr(SO<sub>3</sub>H)-pNle-mGly-Trp-Me(Nle)-Asp-PheNH<sub>2</sub>] is a highly potent (0.15 nM) and selective agonist for CCK-B receptors which cross the blood brain barrier. Following i.v. injection of [3H]pBC 264 in mouse, the ligand was found in its intact form in brain tissue. Analgesic studies and in vivo binding experiments have shown that the CCKergic system could modify the release of endogenous enkephalins, whereas mu and delta opioid receptor activation modulates the release of endogenous CCK. Behavioural studies performed after local injection of CCK-8 or BC 264 into the postero-median part of the nucleus accumbens have shown the involvement of CCK-A receptors in motivation and/or emotional states of rats. In the anterior part, CCK-B receptor stimulation could be involved in attention and memory processes. BC 264 systemically administered in mice increased fear and/or "anxiety" in the black and white box test. In the elevated plus maze, BC 264 increased the emotional responses of the "anxious" rat and decreased these responses in "non anxious" animals. These results suggest that endogenous CCK could play a critical role in mood modulation through CCK-A/CCK-B receptor stimulation. Dysfunctioning of the CCK-A/CCK-B pathways could be implicated in anxiety and panic attacks.

PMID: 1301645 [PubMed - indexed for MEDLINE]

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Stimulation of delta-opioid receptors reduces the in vivo binding of the cholecystokinin (CCK)-B-selective agonist [3H]pBC 264: evidence for a physiological regulation of CCKergic systems by endogenous enkephalins. [J Neurochem. 1992] PMID: 1357099

Investigation of behavioral and electrophysiological responses induced by selective stimulation of CCKB receptors by using a new highly potent CCK analog, BC 264. [Synapse. 1990] PMID: 2399491

In vivo binding affinities of cholecystokinin agonists and antagonists determined using the selective CCKB agonist [3H]pBC 264. [Eur J Pharmacol. 1991] PMID: 1797561

The CCK-B agonist, BC264, increases dopamine in the nucleus accumbens and facilitates motivation and attention after intraperitoneal injection in rats. [Eur J Neurosci. 1997] PMID: 9383203

[3H]pBC 264, a suitable probe for studying cholecystokinin-B receptors: binding characteristics in rodent brains and comparison with [3H]SNF 8702. [Mol Pharmacol. 1992] PMID: 1614411

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